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STATUS SOCIAL E METABOLISMO AERÓBICO EM *Apistogramma agassizii* e *A. hippolytae* (Perciformes: Cichlidae)

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STATUS SOCIAL E METABOLISMO AERÓBICO EM *Apistogramma agassizii* e *A. hippolytae* (Perciformes: Cichlidae)

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Sinopse

Este estudo teve como objetivo entender as relações entre metabolismo aeróbico e a posição na hierarquia social em *Apistogramma* spp em ambiente natural e em diferentes situações experimentais. O estudo das relações sociais e da taxa metabólica de *A. agassizii* em ambientes com diferentes graus de complexidade da estrutura ambiental foi realizado. Também foram estudadas como mudanças nas variáveis parâmetros ambientais (no caso, temperatura e concentração de oxigênio) influenciam as relações sociais, os parâmetros metabólicos e as hierarquias de dominância de *A. agassizii.* Por fim, foram estudadas duas populações naturais de *A. hippolytae* e diferenças comportamentais e fisiológicas foram observadas.

Palavras-chave: hierarquias de dominância, peixes, Cichlidae, taxa metabólica, *Apistogramma*

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RESUMO

Esse estudo objetivou entender as relações entre metabolismo aeróbico e posição hierárquica em espécies do gênero *Apistogramma* (Cichlidae) e como diferenças ambientais poderiam influenciar essas relações. No primeiro capítulo, verificou-se a influência de mudanças na temperatura e na concentração de oxigênio da água nas interações sociais de grupos de *Apistogramma agassizii*. Também avaliou-se como essas mudanças influenciavam o metabolismo aeróbico da espécie e como os parâmetros comportamentais interagiam com os parâmetros fisiológicos. Observou-se uma redução na estabilidade das hierarquias de dominância de grupos de indivíduos mantidos em aquários submetidos a temperaturas mais elevadas. Aumento da temperatura e diminuição no oxigênio alteraram os níveis de agressão nos grupos de *A. agassizii*. Peixes dominantes em ambiente sem modificações ambientais foram os únicos que tiveram uma vantagem na taxa de forrageamento quando comparados aos subordinados. A taxa metabólica foi maior em peixes dominantes apenas nos aquários sem mudanças ambientais. O segundo capítulo avaliou como diferenças na complexidade estrutural do habitat interferem nas interações sociais de duplas de machos de *A. agassizii*. Além disso, verificou-se como a complexidade estrutural interfere na taxa metabólica dos animais e como esses parâmetros fisiológicos interagiam com os parâmetros comportamentais. Foi observado um aumento na frequência de mordidas desferidas pelos peixes dominantes nos aquários com maior complexidade ambiental. Também foi observado um aumento na taxa metabólica em peixes dominantes quando comparados aos subordinados, mas esse aumento ocorreu apenas nos aquários com maior complexidade ambiental. No terceiro capítulo investigou-se a existência de hierarquias de dominância nas interações sociais de populações naturais de *A. hippolytae* nas Reservas Dimona e Amanã. Além disso, verificou-se a influência do status social no metabolismo aeróbico de indivíduos da espécie. Grupos de *A. hippolytae* observados na Dimona tiveram taxas mais altas de agressividade e de alimentação quando compados aos grupos da Reserva Amanã. Foi observado que peixes dominantes possuem vantagens metabólicas nas duas localidades, porém na reserva Amanã peixes na última posição hierárquica possuem perfis metabólicos semelhantes aos peixes dominantes. Conjuntamente, esses resultados indicam que o status social influencia no metabolismo aeróbico de *A. hippolytae* e *A. agassizii*, e que os fatores ambientais interferem nessa influência.

ABSTRACT

Social status and aerobic metabolism in *Apistogramma agassizii* e *A. hippolytae* (Perciformes: Cichlidae)

This study aimed to understand the relationship between social status and aerobic metabolism in species of *Apistogramma* (Cichlidae) and how environmental differences can affect these relationships. In the first chapter, we evaluated the influence of changes in temperature and oxygen concentration in social interactions of groups of *Apistogramma agassizii*. In addition, we assessed how changes in the environmental parameters influence the aerobic metabolism of this species and how environmental parameters interact with the physiological ones. There was a reduction in stability of dominance hierarchy in the high temperature. Aggression levels changed significantly after the increase in temperature and decrease in oxygen concentration. Dominant fish from undisturbed environment were the only that ate more than their respective subordinates. When comparing metabolic rates in relation to social status, dominant fish had higher metabolic rate than their subordinates only in undisturbed environment. The second chapter evaluated how differences of habitat complexity influences social interaction of pairs of males of *A. agassizi*. In addition, we investigated how habitat structure influences metabolic rate and how physiology interacts with behaviour in differently structured enviornments. We observed an increase in biting by dominant fish at habitat with higher structural enrichment. We observed an increase in metabolic rate in dominant fish only in enriched habitats. In the third chapter the existence of dominance hierarchy in the social interactions in natural populations of *A. hippolytae* at Dimona and Amanã Reserve was investigated. In addition, we evaluated the influence of social status in the aerobic metabolism of this species. Groups of *A. hippolytae* at Dimona site had higher aggressiveness and feeding rate when compared to Amanã site groups. We observed that dominant fish have metabolic advantages in both studied sites; however, in Amanã the most subordinate fish presented aerobic metabolic profile similar to that of dominant one. Altogether, our results show that social status influences aerobic metabolism of *A. hippolytae* and *A. agassizii*, and that environment affects this influence.

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INTRODUÇÃO GERAL

Na natureza, é comum observar animais vivendo em grupos. Entre animais, a competição por algum recurso escasso frequentemente leva à formação de hierarquias sociais de dominância (Whiteman and Côté, 2004). A hierarquia de dominância surge quando membros de um grupo social interagem, frequentemente agressivamente, para formar um sistema de posições sociais, onde os animais que vencem as interações agressivas são ditos dominantes e os que perdem, subordinados (Alcock, 2005). Nessas hierarquias, indivíduos dominantes tomam para si a maior parte dos recursos por meio do uso de agressão, podendo esta ser ritualizada ou não, forçando os animais subordinados a manterem-se em uma postura não agressiva para sua própria segurança (Hollis et al., 2004). Peixes, assim como outros animais, frequentemente desenvolvem hierarquias de dominância social, com visíveis diferenças comportamentais entre indivíduos dominantes e subordinados.

As diferenças observadas em nível comportamental também são documentadas em nível fisiológico, como observado em peixes salmonídeos e ciclídeos. Indivíduos dominantes apresentam geralmente maior peso corporal e maior tamanho, e mantêm uma maior taxa de crescimento (Earley and Dugatkin, 2006; Montero et al., 2009; Sloman et al., 2002a; Sloman et al., 2000a; Sloman et al., 2001; Whiteman and Côté, 2004). Essa maior taxa de crescimento vista em peixes dominantes está geralmente relacionada a uma maior ingestão de alimento, já que os peixes dominantes tendem a monopolizar os recursos alimentares disponíveis (Adams and Huntingford, 1996; MacLean and Metcalfe, 2001; Montero et al., 2009; Whiteman and Côté, 2004). Muitos desses resultados foram produzidos sob condições experimentais controladas em laboratório, mas alguns trabalhos que investigaram hierarquias em ambientes naturais estáveis mostraram esses mesmos resultados (Höjesjö et al., 2002; Nakano, 1995; Sloman et al., 2007). Todos os trabalhos citados acima foram capazes de identificar a formação de hierarquias estáveis, por curto ou longo prazo, em ambiente laboratorial ou natural.

Uma característica fisiológica relacionada com a posição social dentro de uma hierarquia de dominância é a taxa metabólica. Aumentos na taxa metabólica podem estar associados com comportamento agressivo e, consequentemente, com a posição de dominância. Grantner and Taborsky (1998) observaram um aumento na taxa metabólica no ciclídeo *Neolamprologus pulcher*quando os mesmos estavam realizando comportamentos agonísticos. Uma maior taxa metabólica aumentou a probabilidade de tornar-se dominante em indivíduos de *Salmo salar* (Metcalfe, Taylor, & Thorpe, 1995). Em *Oncorhynchus masou*, foi observada correlação positiva entre taxa metabólica e posição de dominância, sendo que quanto maior a taxa metabólica, mais alta a posição hierárquica do indivíduo (Yamamoto, Ueda, & Higashi, 1998). Correlação positiva entre agressão e taxa metabólica também foi observada no ciclídeo *Oreochromis mossambicus* (Ros, Becker, & Oliveira, 2006). Alguns estudos, porém, não reportam relação entre o status social ou agressividade e a taxa metabólica. Seppanen et al. (2009), estudando três populações de *Salmo salar*, não encontraram relações entre a agressividade e a taxa metabólica, apesar de terem encontrado diferenças na taxa metabólica entre as populações. Grobler & Wood (2013) estudaram uma série de parâmetros metabólicos em hierarquias de dominância em *Oncorhynchus mykiss* e não observaram relações entre a taxa metabólica e o status social.

Os estudos mencionados acima relacionando taxa metabólica com status social foram todos realizados em condições controladas e estáveis. Porém, diferenças ambientais podem causar alterações no comportamento dos peixes, com consequências nas suas relações sociais. Kagawa (2013) estudou populações naturais de espécies de medaka japonês (*Oryzias latipes* e *O. sakaizumii*) em cativeiro e observou uma maior agressividade em peixes dominantes originários de populações do sul, quando comparados àqueles originários de populações do norte. Diferenças na agressividade também foram observadas em duas linhagens de *Oncorhynchus mykiss* (Schjolden et al. 2005). Portanto, divergências nas populações podem levar a diferenças no comportamento dos indivíduos, com consequências na sua fisiologia.

A agressividade também pode ser afetada pela complexidade estrutural do habitat (Schoener, 1987). Uma maior complexidade estrutural do habitat pode interferir

na taxa de agressão, primeiramente, reduzindo a taxa de encontro entre os indivíduos e, também, pelo aumento na quantidade de recursos (Barley & Coleman 2010). Essa redução na agressividade com o aumento da complexidade ambiental foi observada em *Archocentrus nigrofasciatus* (Cichlidae) (Barley & Coleman 2010), *Sparus aurata* (Sparidae) (Barreto, Carvalho, & Volpato, 2011; Batzina, Dalla, Papadopoulou-Daifoti, & Karakatsouli, 2014; Kelley, Magurran, & Garcia, 2006) e *Ameca splendens* (Goodeidae) (Kelley et al. 2006). Não há estudos de como essas mudanças comportamentais causadas pelas diferenças na complexidade ambiental afetam o metabolismo aeróbico relacionado ao status social.

A instabilidade ambiental também pode interferir no comportamento social de peixes. Vários estudos têm descrito resultados variáveis em decorrência do efeito de distúrbios ambientais na estabilidade e estrutura social de diversos organismos. Sloman *et al.* (2002b) submeteram grupos de *Salmo trutta* a um aumento no fluxo de água e verificaram que aqueles grupos que eram submetidos a esse distúrbio ambiental tinham seu comportamento social alterado, e as vantagens fisiológicas observadas naqueles peixes dominantes de ambientes estáveis desapareciam. Os mesmos resultados foram observados quando espécimes de *S. trutta* foram submetidos a uma diminuição do nível de água, simulando um evento de seca (Sloman et al., 2001). Sneddon *et al.* (2006) expuseram *Gasterosteus aculeatus* a dois tipos de perturbações ambientais (aumento do fluxo de água e diminuição nos níveis de água) e observaram uma diminuição na estabilidade das hierarquias sociais, bem como mudanças nos níveis de agressão nos grupos de peixes submetidos às referidas perturbações. Esses mesmos autores concluíram que as condições ambientais têm efeitos significativos na estrutura e estabilidade das hierarquias naquela espécie. Dessa maneira, os parâmetros fisiológicos e, entre eles, a taxa metabólica, relacionados ao status social podem ser afetados por mudanças ambientais.

Nesse contexto, os peixes da família Cichlidae são objeto frequente de estudos devido à complexidade comportamental dos mesmos, gerando informações importantes para o entendimento das funções e causas do comportamento (Hofmann & Fernald, 2001. As espécies do gênero *Apistogramma* formam hierarquias de

dominância, possuem cuidado parental elaborado e muitas espécies apresentam dimorfismo sexual evidente (Axelrodi, 1993; Kullander, 2003; Römer and Beisenherz, 2005; Römer and Beisenherz, 1996). Além disso, diversas espécies do gênero ocupam habitats de águas claras em corpos d'água rasos, o que facilita a observação de seu comportamento no campo. Apesar de diversos estudos comportamentais com espécies do gênero terem sido realizados (Romer 1995, 1998, 2005, Rodrigues et al. 2012), até onde foi possível constatar, investigações sobre as relações entre status social e metabolismo aeróbico não foram realizados. Além disso, por serem de pequeno porte e de fácil manuseio e manutenção em cativeiro, as espécies desse gênero possuem potencial para se tornarem modelos de estudos comportamentais entre os ciclídeos Neotropicais.

Na presente tese, apresentada a seguir em três capítulos, procuro entender as relações entre o status social e o metabolismo aeróbico em duas espécies do gênero *Apistogramma* (*A. hipollytae* e *A. agassizii*) em diferentes contextos sociais e ambientais. No primeiro e segundo capítulos, *A. agassizii* foi utilizada como modelo para estudos desenvolvidos sob condições laboratoriais. No capítulo um, investiguei como a instabilidade ambiental afeta o comportamento social e os parâmetros metabólicos relacionados ao status social, manipulando a temperatura e a concentração de oxigênio em aquários mantidos em ambiente controlado. No capítulo dois, estudei o efeito de variações na complexidade ambiental sobre o comportamento social da espécie, e como a complexidade ambiental interfere nas relações entre o status social e a taxa metabólica. No terceiro capítulo, a espécie *A. hipollytae* foi estudada em ambientes naturais em duas localidades do estado do Amazonas, a fim de verificar se haveria diferenças comportamentais entre as populações, e se essas diferenças levariam a modificações nos parâmetros metabólicos relacionados ao status social.

OBJETIVOS

O objetivo geral foi:

Entender as relações entre metabolismo aeróbico e a posição hierárquica em grupos de indivíduos de duas espécies do ciclídeo-anão *Apistogramma* em ambiente natural e em diferentes situações experimentais em cativeiro.

Os objetivos específicos de cada capítulo foram:

Capítulo I: Verificar a influência da temperatura e da concentração de oxigênio dissolvido nas interações sociais e no metabolismo aeróbico, bem como a relação entre esses parâmetros em *Apistogramma agassizii.*

Capítulo II: Verificar como diferenças na complexidade estrutural do habitat interferem nas interações sociais e, na taxa metabólica de *Apistogramma agassizii* e como estes parâmetros se relacionam entre si.

Capítulo III: Verificar a influência de parâmetros ambientais nas interações sociais e no metabolismo aeróbico, bem como a relação entre esses parâmetros em populações naturais de *Apistogramma hippolytae.*

Capítulo I

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Experimentally increased temperature and hypoxia affect stability of social hierarchy and metabolism of the Amazonian cichlid *Apistogramma agassizii*

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Abstract

The primary goal of this study was to understand how changes in temperature and oxygen could influence social behaviour and aerobic metabolism of the Amazonian dwarf cichlid *Apistogramma agassizii*. Social hierarchies were established over a period of 96h by observing the social interactions, feeding behaviour and shelter use in groups of four males. In the experimental environment, temperature was increased to 29°C in the high temperature treatment, and oxygen lowered to 1.0 $mg.L^{-1}$ O_2 in the hypoxia treatment. After the stabilization period, fish were maintained at this control condition for 96h to allow hierarchies to stabilise. Control condition was maintained at 26°C and 6.6 $mg.L^{-1}$ O_2. After the experimental exposure, metabolism was measured as routine metabolic rate (RMR) and electron transport system (ETS) activity. There was a reduction in hierarchy stability in the high temperature. Aggression levels changed after environmental changes. Dominant and subdominant fish at high temperature increased their biting, compared to dominant fish from control. In contrast, dominant fish exposed to hypoxia decreased their aggressive acts compared to all other fish. Shelter use decreased in dominant fish under control and hypoxic conditions. Dominant fish from undisturbed environment eat more than their subordinates. There was a decrease of RMR in fish exposed to the hypoxic environment when compared to fish from control or high-temperature environment independently of social position. Dominant fish has higher RMR than their subordinates at the control condition. ETS activity increased in fish exposed to high temperature; however, there was no effect on social rank. Our study reinforces the importance of environmental changes for maintenance of hierarchies and their characteristics and highlight that most of the changes occurs in the dominant position.

Key works: social hierarchy, fish behaviour, Cichlidae, aerobic metabolism, metabolic rate, aggressiveness

1. Introduction

Competition for resources often leads to the formation of a dominance hierarchy which is determined through aggression in living animal groups (Höglund et al., 2001; Whiteman and Côté, 2004). Dominant individuals control most of the resource whereas subordinate animals are forced to maintain a non-aggressive behaviour and have access to reduced resources (Hollis et al., 2004).

Differences in behaviour are in general followed by differences in physiological status of the animal (Metcalfe et al., 1995). Dominant fish are bigger and maintain a higher growth rate (Earley and Dugatkin, 2006; Montero et al., 2009; Sloman et al., 2002a; Sloman et al., 2000a; Sloman et al., 2001; Whiteman and Côté, 2004). This high growth rate is related to higher food ingestion, as dominant fish can monopolize food (Adams and Huntingford, 1996; MacLean and Metcalfe, 2001; Montero et al., 2009; Whiteman and Côté, 2004). One of the most reported physiological consequences of dominant behaviour are higher metabolic rates (Grantner and Taborsky, 1998; Metcalfe et al., 1995; Vollestad and Quinn, 2003; Yamamoto et al., 1998). The higher metabolic rate of dominant fish seems to be the way they achieve higher growth rates (Hoogenboom et al., 2013; Millidine et al., 2009a).

Most of the studies investigating physiological consequences of dominance hierarchies were carried out in stable environments. However, natural environment is often unstable. Sneddon et al. (2006) investigating social behaviour of three-spined sticklebacks, *Gasterosteus aculeatus*, observed a decrease in hierarchy stability when water level and turbulence were changed. The levels of aggression were higher after the water level was dropped. A decrease in the water level also affected stability and benefits of dominance in *Salmo truta* (Sloman et al., 2001).

Temperature also has an influence on fish behaviour. Biro et al. (2010) observed differences in activity, aggressiveness and boldness in damselfish as they were exposed to different temperatures. They suggest that individual differences in energy metabolism may contribute to animal personality, given that the temperature has large direct effects on metabolic rates in ectotherms. So, we design this study aiming to

understand how changes in temperature and oxygen, another key parameter affecting energy metabolism, can influence social behaviour in the dwarf cichlid *Apistogramma agassizii*. Metabolism was measured in terms of resting metabolic rate (RMR) and through electron transport system (ETS) activity. RMR is considered the minimal respiration rate of fasting animal at certain temperature, performing minimal movements (Lampert, 1984). ETS activity is often used to estimate metabolic potential for various groups of organisms as the amphipod *Gammmarus fossarum* (Simčič and Brancelj, 2003), the noble crayfish *Astacus astacus* and the fish *Salmo marmoratus* (G.-Tóth et al., 1995; Simčič et al., 2015). ETS is considered a biochemical measurement of the metabolic potential of the animal, i.e. the oxygen consumption that would occur if all enzymes of the multienzyme complex of respiratory chain function at maximum rates (Muskó et al., 1995).

The species *Apistogramma agassizii* is a representative of the South Amercian dwarf cichlids. The genus *Apistogramma* Regan (1913) comprises 84 valid species (Schindler and Staeck, 2013) making the genus *Apistogramma* one of the most diverse genera of the Cichlidae family. *A. agassizii* is found in clear and black water streams, locally known as igarapés, across the Amazon River basin, along Amazon-Solimões River from Peru through Brazil to Capim River basin (Kullander, 2003). Temperature and dissolved oxygen concentration in the forest streams are in the range of 24.4-26.2°C and 3.5-9.2 $mg.L^{-1}$ O_2 (Rodrigues et al. 2012; Carvalho et al. 2014). However, some of these forest streams can have very low oxygen concentration. The species can be collected even in habitats with oxygen as low as 0.05 $mg.L^{-1}$ (Hercos et al. 2009).

2. Materials and Methods

2.1 Fish and holding conditions

A. agassizii has clear sexual dimorphism. Specimens used in our study were collected in streams close to Tefé Lake (03°29'3561''S 64°76'9832''W), near the city of Tefé, transferred to plastic bags filled with water, inflated with oxygen and transported by boat to the Laboratory of Ecophysiology and Molecular Evolution at the Brazilian

National Institute for Research in the Amazon (INPA), in Manaus where the experiments were carried out. In the lab, the fish were divided between four large stock aquaria (500L), containing dead leaves and some trunks and twigs to simulate their natural habitat. They were maintained in continuously aerated tanks for at least 3 weeks before starting the experiments, under a natural photoperiod (12:12 h light:dark). Water temperature, pH and dissolved oxygen were 26.4±0.7°C, 6.5±0.3, 6.2±0.5 $mg.L^{-1}$ O_2, respectively. Fish were fed commercial food pellets containing 48% of protein, twice daily. All fish procedures followed INPA's animal care guidelines and were approved by INPA's animal care committee.

2.2 Fish tagging

For behavioural observations, fish from the holding tanks were tagged to allow individual recognition using a visible elastomer implant tags (Northwest Marine Technology Inc.) following the standardized protocol recommended by the manufacturer. Using a syringe (29 gauge, 0.3 cc), a small bolus of elastomer of one of four colours (green, pink, yellow and blue) was injected by the dorsal fin, right and left side. All tagged fish were then wet weighed (0.59±0.08 g) and had the standard length measured (24.08±2.40 mm). Fish were handled without the use of anaesthetics because they can interfere with physiological analysis and also, when tested, the time to resume normal behaviour was longer after anaesthesia than without anaesthesia. Tagging, weighing and measuring took less than 1 min and fish returned to normal behaviour one hour after handling. After tagging, fish were held in one out of four aquaria, one for each colour, to give a time to ensure tag retention. Only male fish were tagged to avoid introducing courtship behaviour in the experiment (Cole et al., 1980).

2.3 Behavioural observations

One week after tagging, four fish, each one with a different tag colour, were selected from the recovery tank and transferred to an experimental aquarium (60 X 35 X 25 cm; 50 L) so that behavioural observations could be made to determine dominance

relationships. All fish were introduced into the experimental arena at the same time to avoid effects of prior residence. Each aquarium had a plastic tube (2 cm diameter, 10 cm long) as a shelter, a thermostat to control temperature and an air stone on one side of the aquarium to aerate the water. The four fish in each aquarium were from different holding tanks and were randomly selected to avoid familiarity and previous hierarchical effects. Groups of four fish were randomly assigned to the different experimental conditions (see Experimental Manipulations below) and were given 24 h to acclimate to the tanks and to establish a dominance hierarchy. The behaviour of the fish was monitored for a 30-min period every day during the experimental period. During this period, we recorded the number of chases (bursts of increased swimming speed directed at other individuals), the number of bites, and the number of tail beats towards another fish. After 20 min of recording, fish were fed a standardized amount of food (20 food pellets of similar size). The food was always placed in front of the shelter to record feeding behaviour. For feeding behaviour, we recorded the number of feeding bites of each fish. The number of times that fish used the shelter was also counted. All observations occurred between 13:00 to 16:00 h to control for temporal variation in the activity level of the fish. We measured dominance by assigning points on a weighted scoring system similar to the one used by Sloman et al. (2001). Each fish was scored according to its shelter use, its consumption of food and its social interactions (Table 1). All scores were summed which generated an overall score for each fish (fish score). We could then rank each fish according to its fish score, where rank 1, with the highest fish score, was classed as the dominant fish, rank 2 the subdominant, and ranks 3 and 4 as subordinate fish.

2.4 Experimental manipulations

The experiments consisted of two different treatments involving manipulation of water temperature and oxygen and an undisturbed control treatment that was kept at 26±0.5°C and under normoxia (6.6±0.3 $mg.L^{-1}$ O_2). Aquaria were kept in control conditions for 96h before conditions were changed for the temperature and hypoxia treatment for another 96h. This experimental design ensured that any changes in

dominance behaviour were because of the change in environmental conditions and not simply because of the time course of the experiments (Sneddon et al., 2006). For the analysis of the effects of temperature, the temperature was raised from 26°C to 29°C over a period of 2h. For the analysis of hypoxia, we lowered oxygen concentration to 1.0 $mg.L^{-1}$ O_2 by injecting nitrogen through the air stone. Oxygen concentration decreased from normoxia to 1.0 $mg.L^{-1}$ O_2 over a period of 2h. Low oxygen concentration was maintained by covering aquaria with transparent plastic film and bubbling nitrogen gas. Each treatment were replicated in 8 aquaria (n=8).

At the end of the experiment, each fish was removed from the tank, weighed and measured before determination of their RMR, and ETS.

2.5 Physiological measurements

2.4.1 Resting metabolic rate (RMR)

After behavioural observations, RMR was measured as oxygen consumption using an intermittent flow respirometer. RMR was calculated by measuring the reduction in dissolved oxygen, flowing past a stationary fish (Steffensen, 1989). Water temperature and oxygen were kept stable over the experimental period: control at 26±0.1°C and normoxia (6.7±0.3°C $mg.L^{-1}$ O_2), high temperature at 29±0.1°C and normoxia (6.5±0.4 $mg.L^{-1}$ O_2), and hypoxia (1.4±0.3 $mg.L^{-1}$ O_2) and 26.1±0.1°C. We used a computerized apparatus which consists of a recirculation circuit and a 70 mL respiration chamber. The flow in flush time was as low as possible to achieve replacement of water without disturbing the fish. Feeding was suspended 24h before the measurement to allow the fish to empty their guts. Dissolved oxygen and temperature were automatically monitored and controlled by DAQ-M (Loligo Systems, Tjele, Denmark). Calibration of the oxygen electrode was achieved with air-saturated water from the header tank as 100% saturation and a solution of sodium sulphite as 0%. Peristaltic pumps were used to flush the chambers with water from the ambient tank during the flush phase and were stopped during the measurement phase. The phase times were 120s flush followed by 180s wait and 600s measurement. Thus, the duration of an entire measurement ‘loop’ was 15 min. The oxygen measurement in the chambers

was performed across sensor spots, stacked inside the chambers, and optical fiber cables connected to OXY-4 or Witrox 4 (Loligo Systems). Fish were allowed to acclimate to the respirometer for 2h before starting the measurements. Metabolic rate was monitored over a 3h period (12 measurements per fish) and a mean value was calculated. Oxygen consumption rate, MO_2 ($mg.O_2.kg^{-1}.h^{-1}$), was calculated as $MO_2 = -\Delta OV resp\ B^{-1}$, where O is the rate of change in oxygen tension ($kPa\ h^{-1}$), Vesp is the volume of the respirometer, and B is the individual mass (kg). We also measured microbial consumption in empty chambers and the values of metabolic rate were corrected for this background.

2.4.2 Electron transport system (ETS) measurements

After measurement of RMR, fish were used for ETS measurements. Fish were identified, weighed, measured, rapidly euthanized and immediately transferred to a liquid nitrogen container for measurements of electron transport system activity (ETS). ETS was measured using the method proposed by Packard (1971) and improved by G.-Tóth (1993). The solutions were prepared 24h before use and maintained on ice to avoid substrate decomposition and bacterial contamination. The whole animal was homogenised with liquid nitrogen using a mortar and a pestle. The pre-weighed homogenised material (30-60 mg wet mass) was then sonicated, using a sonifier (VWR Scientific, model 450, USA) in 4 mL of ice-cold homogenisation buffer (0.1M sodium phosphate buffer pH = 8.4; 75 µM $MgSO_4$; 0.15%(w/v) polyvinyl pyrrolidone; 0.2%(v/v) Triton-X-100) for 20 s and centrifuged for 4 min at 0°C at 8500*g* using a Eppendorf centrifuge 5430R (Eppendorf, Germany). Three 0.5-mL samples from each homogenate were incubated in 1.5 mL substrate solution (0.1M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2%(v/v) Triton-X-100) with 0.5 mL 2.5 mM 2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyl tetrazolium chloride (INT) solution for 20 min at the same temperature that RMR was measured (27°C at Dimona and 25°C at Amanã). The reaction was stopped by adding 0.5 mL of 1:1 formalin:H_3PO_4. Blanks (1.5 mL substrate solution and 0.5 mL INT solution) were incubated and stopped as for the samples; 0.5 mL of homogenate was added after the reaction was stopped. Formazan

production was determined spectrophotometrically from the absorbance of the sample at 490 nm against the blank, within 10 min of stopping the reaction, using a spectrophotometer Spectramax Plus 384 (Molecular Device, USA). The ETS activity of each animal was determined using the procedure described in Simčič & Brancelj (2003). ETS activity was measured as the rate of tetrazolium dye reduction to formazan and converted to equivalent oxygen consumed per mg of wet mass per hour ($\mu LO_2.mgWW^{-1}.h^{-1}$) as described by Kenner & Ahmed (1975).

3. Statistical analyses

To obtain the dominance stability value, we used Kendall's Coefficient of Concordance as in Sneddon et al. (2006) to test how concordant the dominance score was for each rank within a hierarchy. Briefly, Kendall's Coefficient of Concordance gave a coefficient value between 0 and 1, 0 meaning no stability and 1 meaning totally stable. This was performed using the dominance score for each of the four fish per tank at 24h against the dominance score of each of the four fish per tank at the first 96h period (pre manipulation) and comparing this with the second 96h period (post manipulation). A decrease in the value indicated a reduction in stability and an increase in the value indicated an increase in stability. A two-factor ANOVA was used to assess if the coefficient values were significantly different before and after the manipulation and also between treatments.

To determine behavioural changes, we deducted the mean number of aggressive acts and shelter use of the second 96h period (after change) from the first 96h period (before change) and ran a two-factor ANOVA (environmental condition and social status) for each behavioural parameter. To assess differences in monopolization of food between treatments, feeding behaviour was assessed by a two-factor ANOVA with social position and treatment (control, increase temperature or hypoxia) as factors and the mean of feeding bites within the second 96h period (after change) as a dependent variable. A two-factor ANOVA was used independently to assess relationships between physiological parameters and behavioural data, with treatment and social position as

factors and RMR or ETS activity as dependent variables. Data for these analyses were log+1 transformed to achieve a normal distribution. Data are shown as mean±SEM.

4. Results

Fish scores before changes in environment conditions easily identified dominance hierarchies. Dominant individuals were evident in all treatments (Fig. 1). Social ranks were stable in all treatments before manipulation, with high Kendall's Coefficient of Concordance and no differences between treatments. However, there were changes in the stability of the dominance hierarchies after environmental manipulation. There was a reduction in stability in the high-temperature treatment (Fig. 2; Two-factor ANOVA, before and after effects, $F_{1,24}$=11.7050; p=0.0022). There was a significant effect between treatments ($F_{2,24}$=6.2465; p=0.0065) and also an interaction between treatment and before and after effects on stability of the dominance hierarchies ($F_{2,24}$=9.4424; p=0.00094) (Fig. 2).

Aggression levels changed significantly after environmental changes ($F_{2,48}$=6.9492; p=0.002). However the changes were dependent on social rank ($F_{3,48}$=5.3811; p=0.004) and there was an interaction effect between treatment and social rank ($F_{6,48}$=4.1440; p=0.001). Dominant and subdominant fish at the increased temperature increased significantly their aggressiveness when compared to dominant fish from the undisturbed environment, but not when compared to subordinate ranks at same treatment. Dominant fish exposed to lowered oxygen levels showed a decrease in their aggressive acts compared to all other fish. There was no difference in aggressiveness changes between all ranks in the undisturbed environment (Fig. 3). Shelter use also changed between the first and second period of 96h. There was no effect of treatment ($F_{2,48}$=2.7537; p=0.07) but there was an effect of social rank ($F_{3,48}$=5.0077; p=0.004) and an interaction effect of social rank and treatment ($F_{6,48}$=3.2000; p=0.01). Shelter use decreased significantly in dominant fish under control and hypoxic conditions when compared to all other fish (Fig. 4).

285 Feeding behaviour was different between treatments ($F_{2,48}$=4.5770; p=0.01) and
286 between social ranks ($F_{3,48}$=2.8534; p=0.04) but there was no interaction effect
287 ($F_{6,48}$=1.2456; p=0.30). Dominant fish from the undisturbed environment showed an
288 increase in feeding, compared to all other ranks (Fig. 5).

289 Environmental change affected RMR ($F_{2,48}$=42.505; p<0.001). There was a
290 decrease in RMR of fish exposed to the hypoxic environment when compared to the
291 undisturbed or high-temperature environment (Fig. 6). However, by two-way ANOVA,
292 there was no effect of social rank on RMR ($F_{3,48}$=0.8721; p=0.46) or an interaction effect
293 of social rank and environmental condition ($F_{6,48}$=0.4909; p=0.811). As we observed a
294 tendency for higher RMR in dominant fish from the undisturbed environment, we ran a t-
295 test comparing dominant individuals against all subordinates. The RMR of dominant fish
296 was higher than their subordinates (t_{18}=3.116; p=0.006) (Fig. 6). ETS activity also
297 changed with environmental changes ($F_{2,48}$=42.505; p<0.001). ETS activity increased in
298 fish exposed to an increase in temperature when compared to fish under control
299 (undisturbed environment) and hypoxic conditions (Fig. 7). However, there was no
300 effect of social rank on ETS activity ($F_{3,48}$=0.7420; p=0.53) nor an interaction effect of
301 social rank and environmental condition ($F_{6,48}$=0.1330; p=0.99) (Fig. 6).

302

303 5. Discussion

304 Under constant environmental conditions, dominance hierarchies of the cichlid
305 *Apistogramma agassizii* were stable, with no change in positions in the social rank, with
306 Kendall's coefficient of concordance at maximum when we compare the first 96h with
307 the end of the experiment. Fish exposed to lowered oxygen do not achieve their
308 maximum stability after change. In contrast, the increase in temperature causes
309 instability at dominance hierarchies. We observed a significant reduction in Kendall's
310 coefficient from the first period of 96h when we compared to the second period of 96h
311 (after change). Instability at dominance hierarchies caused by environmental
312 disturbance was observed in previous studies. Sneddon et al. (2006) submitted groups
313 of four three-spined sticklebacks (*Gasterosteus aculeatus*) to either turbulence or

decrease in water levels and observed a decrease in stability of social ranks in both conditions when compared to undisturbed groups. Sloman et al. (2001) submitted groups of brown trout, *Salmo trutta* to a simulated drought and compared stability of dominance hierarchies observing also an increase in instability of hierarchies. This increase in instability was also observed when brown trout hierarchies was submitted to an increase in flow rates (Sloman et al., 2002b). All these studies are in agreement with our results, i.e., changes of environmental conditions promote instability of social hierarchies.

Although Sneddon et al. (2006) observed an overall increase in aggressiveness at the simulated drought treatment, they do not evaluate in which social rank it occurred. In our study, main behavioural changes occurred in the dominant rank. We observed that dominant and subdominant fish from the higher temperature treatment significantly increased their aggressive acts when compared to dominant fish from undisturbed conditions and with dominant fish from hypoxic conditions. Moreover, dominant fish from hypoxic conditions had a decrease in their aggressive acts when compared to the other two groups (control and increased temperature). An increase in aggressiveness was also observed in damselfish (*Pomacentrus bankanensis*) submitted to a small increase in temperature (Biro et al., 2010). Grantner and Taborsky (1998) observed an increase in metabolic rate in the cichlid fish *Neolamprologus pulcher* when performing either aggressive or submissive behaviour. So, decrease in aggressive acts observed either in undisturbed or hypoxic environment could contribute to a reduction in energy expenditure, whereas fish in higher temperature will face an increase in their energetic costs. A decrease in shelter use was also observed in dominant fish from undisturbed and hypoxic environments. This could be a response to stability in dominance hierarchy. Even though we observed a small, not significant, decrease in stability in dominance hierarchy, there was no change in the dominant position.

An expected advantage of dominant position is a monopolisation of food resources (e.g. MacLean and Metcalfe, 2001; Montero et al., 2009; Wong et al., 2008). In our study, only dominant fish from the undisturbed environment had a significant feeding advantage when compared to their subordinates. Differences in feeding rate reinforce the dominant position in a hierarchy (Ang and Manica, 2010). So, the lost of

345 this advantage in dominant fish under high temperature can be considered as a
346 consequence of the instability of the dominance hierarchy. Moreover, the lost of these
347 advantages combined with the increase in aggressiveness and the consequent increase
348 in energetic cost could cause metabolic disturbances in these fish. Fish under hypoxia
349 almost stop feeding. This result is in agreement with Bernier and Craig (2005) and
350 Tran-Duy et al. (2012) that observed a reduction in feed intake respectively in rainbow
351 trout and Nile tilapia exposed to a hypoxic environment. Tran-Duy et al. (2012) argued
352 that the decrease in feed intake is an attempt to reduce energy requirement for
353 maintenance as dissolved oxygen declines. Together with the decrease in
354 aggressiveness observed in dominant fish we propose that *A. agassizii* have adjusted
355 their metabolism to hypoxic condition in order to decrease energetic costs.

356 We would expect that all these behavioural differences between dominants and
357 their subordinates would lead to physiological differences. However, despite the
358 behavioural differences, only minor differences were found in metabolic parameters
359 between social ranks. Dominant fish from undisturbed environment had a higher
360 standard metabolic rate when compared with their subordinates. Higher metabolic rates
361 are often observed in dominant, aggressive fish. Aggressive behaviour was correlated
362 to oxygen consumption in Nile tilapia (Alvarenga and Volpato, 1995). Ros et al. (2006)
363 observed a correlation between aggressive behaviour and oxygen consumption in the
364 cichlid fish *Oreochromis mossambicus* to name just a few. McMarthy (2001) studied
365 juvenile of rainbow trout, measuring their metabolic rates before social interaction and
366 observed that the higher the relative metabolic rate of a fish compared to its opponent,
367 the greater the probability of being dominant. So, higher metabolic rate are an expected
368 characteristic in dominant fish. However this was not observed in any of the analysed
369 groups exposed to disturbed environments. Fish from either the high temperature or
370 hypoxic environments did not show differences between social ranks. As temperature
371 are strongly related to metabolic rate in ectotherms (as reviewed by Clark et al., 2013)
372 we would expect an increase in metabolic rate for fish at higher temperature treatment;
373 however, this was not observed; we observed an increase in the variability in RMR,
374 instead. This could be due to the fact that this temperature is close to the critical
375 temperature for this species. A reduction in RMR is in general expected in animals close

to critical temperature (Cucco et al., 2012). Even though *A. agassizii* are typical from tropical shallow water, the temperature of their habitats is normally around 27°C (Kochhann D., personal observation) and they seem not adapted to higher temperatures. In contrast, fish exposed to hypoxic environment decreased their RMR, an expected result. As pointed before, fish in hypoxic environment try to reduce their basal metabolic costs (Tran-Duy et al., 2012). Amazonian environments are typically hypoxic (Val and Almeida-Val, 1995) and this is also valid for the habitats of *A. agassizii*. Even though forest streams have higher oxygen concentrations than main rivers, *A. agassizii* from our study were collected in streams with low dissolved oxygen, regularly less than 1 $mg.L^{-1}$ O_2. So, it is possible to see this as an adaptive response to reduce metabolic costs.

The activity of the respiratory electron transport system (ETS) is a biochemical measure of the potential metabolic activity (Lampert, 1984b), i.e., is the measurement of oxygen consumption if all enzymes function at their maximum. We hypothesised that dominant fish would have higher ETS activity, but we did not found any difference in ETS activity between ranks. However, Kochhann (unpublished data) found a positive correlation between ETS activity and aggressiveness in *Apistogramma hippolytae*, a closely related species, in the field. Long-term studies should help to better understand the relationship between ETS activity and social rank in *Apistogramma* species. The only change that we observed was an overall increase in ETS activity in fish at the higher temperature environment, an expected result as ETS activity is sensitive to temperature increase (Simcic and Brancelj, 1997). We would also expect a reduction in ETS activity in fish exposed to hypoxia, which did not occur. As ETS activity is a potential measurement, the absence of this decrease can be seen as an adaptive response, preparing for an increase in metabolism when oxygen levels are recovered.

In conclusion, our study reinforces the importance of environmental stability for maintenance of hierarchies. The environmental changes had a significant effect on the characteristics and stability of the dominance hierarchies. Even though the effects of environmental disturbances on stability was already studied (Sloman et al., 2001; Sloman et al., 2002b; Sneddon et al., 2006), our study was the first to ascertain the

social position affected by environmental changes, showing that dominant position was more prone to changes related to environmental disturbances. Natural environment is normally unstable, experiencing frequent changes in temperature and oxygen, among other parameters. Thus, the benefits of dominant position may be overestimated. Future studies should assess how changes in other environmental conditions can impact fish behaviour and, consequently the dominance hierarchies. Also, long-term laboratory studies will help to understand how transient are these responses to environmental perturbations.

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532

533 Table 1. Scoring system used to measure dominance of *Apistogramma agassizii*.

534

Behaviour	Score at each observation
Aggression	
Avoiding another fish	0
Chasing another fish	1
Bitten by another fish	0
Biting another fish	1
Receive a tail beat of another fish	0
Give a tail beat in another fish	1
Feeding	
Fish that failed to get food item	0
Each consumed food item	1
Shelter use	
Fish that did not enter in the shelter	0
Each time fish enter the shelter	1

535 Adapted from Sloman et al. 2001

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538 Figure captions

539

540 **Figure 1.** Mean fish score of individuals of *Apistogramma agassizii* in the first 96h
541 (before change) in each treatment.

542 **Figure 2.** Stability in dominance hierarchies of *Apistogramma agassizii* measured by
543 Kendall Tau coefficient of Concordance for control (no disturbance), increased
544 temperature and lowered oxygen. Different letters means significant difference by two-
545 way ANOVA and Tukey's test. Refer to the text for the detailed statistical analysis.

546 **Figure 3.** Mean change in aggressiveness in dominance hierarchies of *Apistogramma*
547 *agassizii* for control (no disturbance), increased temperature and lowered oxygen.
548 Different letters means significant difference by two-way ANOVA and Tukey's test.
549 Refer to the text for the detailed statistical analysis.

550 **Figure 4.** Mean change in shelter use in dominance hierarchies of *Apistogramma*
551 *agassizii* for control (no disturbance), increased temperature and lowered oxygen.
552 Different letters means significant difference by two-way ANOVA and Tukey's test.
553 Refer to the text for the detailed statistical analysis.

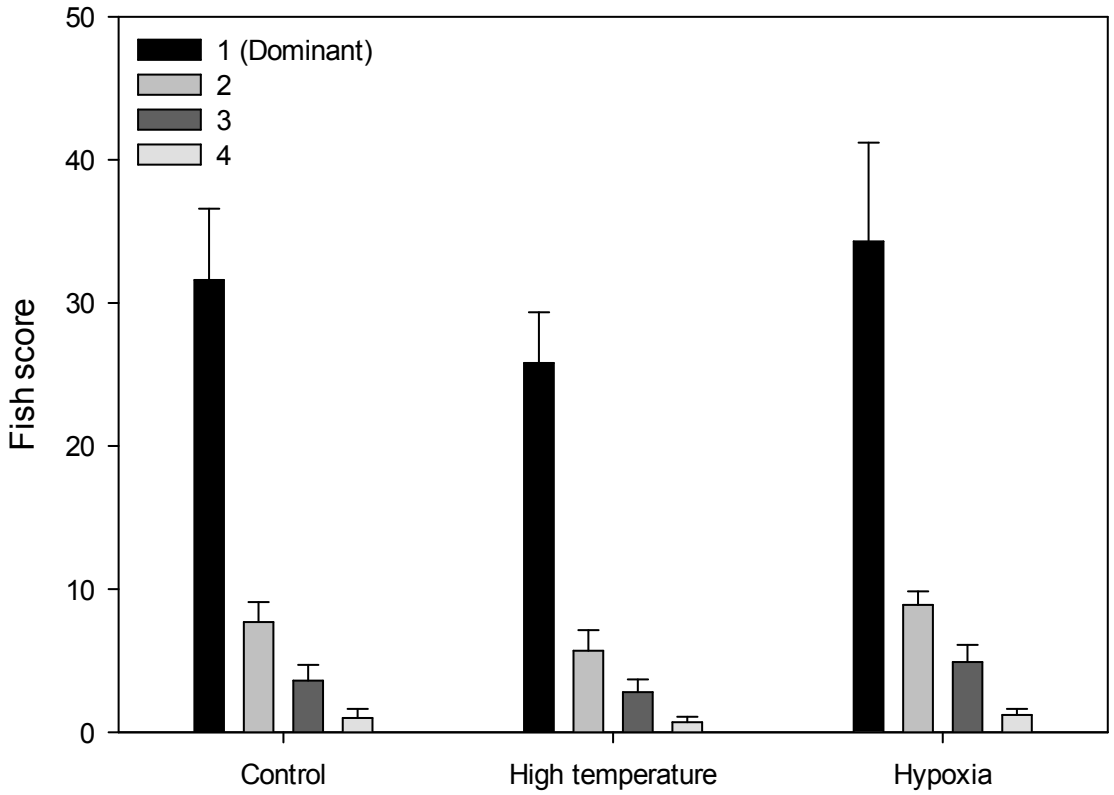
554 **Figure 5.** Mean of feeding bites in dominance hierarchies of *Apistogramma agassizii* for
555 control (no disturbance), increased temperature and lowered oxygen. Different letters
556 means significant difference by two-way ANOVA and Tukey's test. *Significant
557 difference at same treatment by ANOVA. Refer to the text for the detailed statistical
558 analysis.

559 **Figure 6.** Resting metabolic rate (RMR) in dominance hierarchies of *Apistogramma*
560 *agassizii* in no disturbance (control), increase in temperature and lowered oxygen
561 environment. Different letters means significant difference between treatment by two-
562 way ANOVA and Tukey's test. *Significant difference from subordinates by t-test. Refer
563 to the text for the detailed statistical analysis.

564 **Figure 7.** Electron transport system (ETS) activity in dominance hierarchies of
565 *Apistogramma agassizii* for control (no disturbance), increased temperature and
566 lowered oxygen. Different letters means significant difference by two-way ANOVA and
567 Tukey's test. Refer to the text for the detailed statistical analysis.

568

569 Fig. 1



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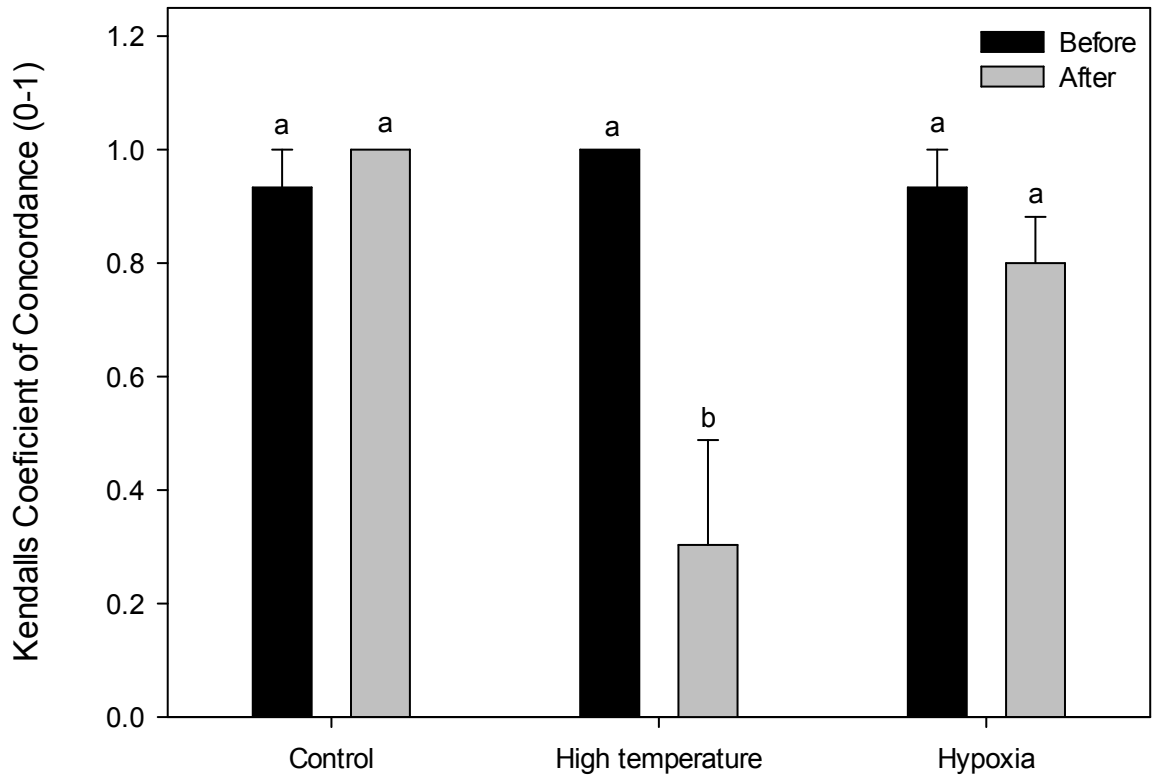
572 Fig. 2



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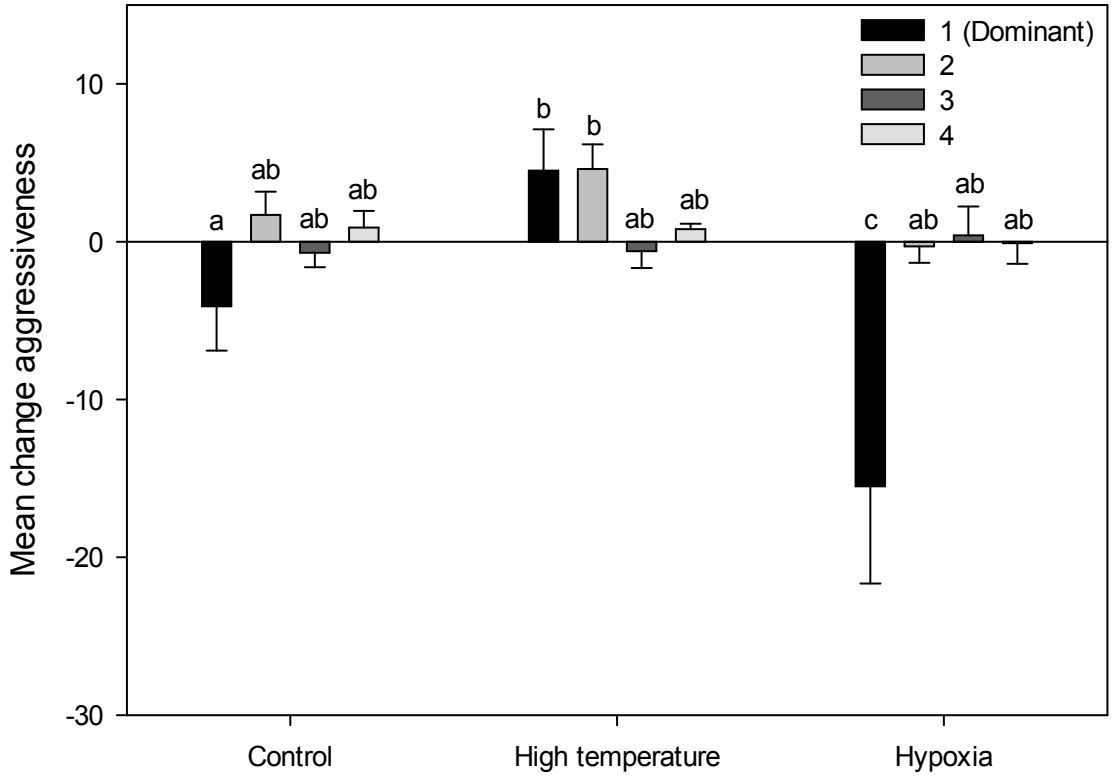
575 Fig. 3



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578 Fig. 4

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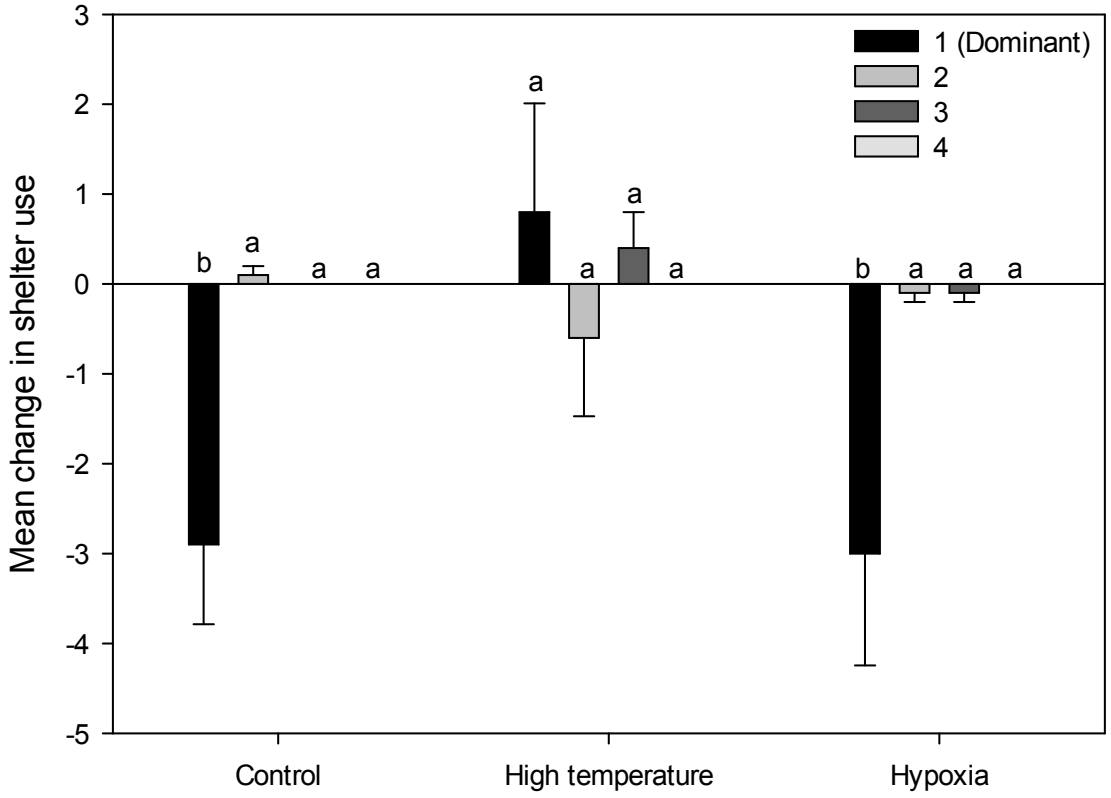

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583 Fig. 5

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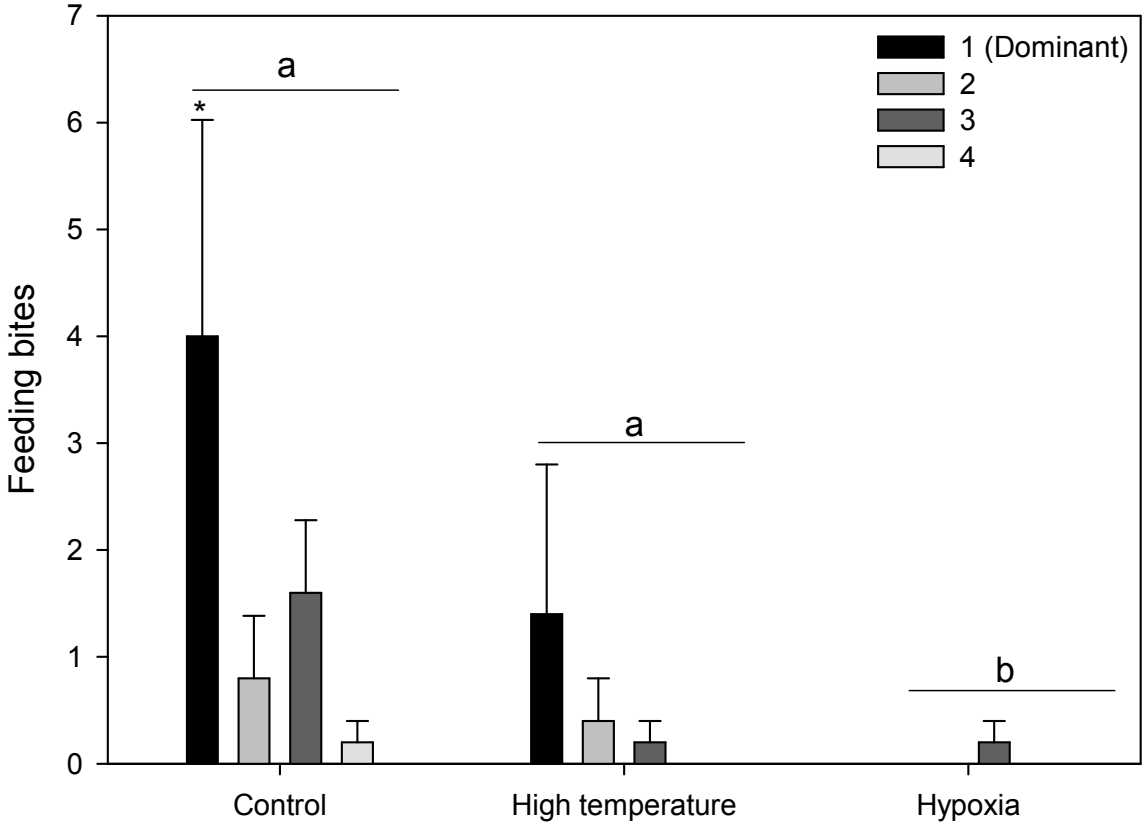

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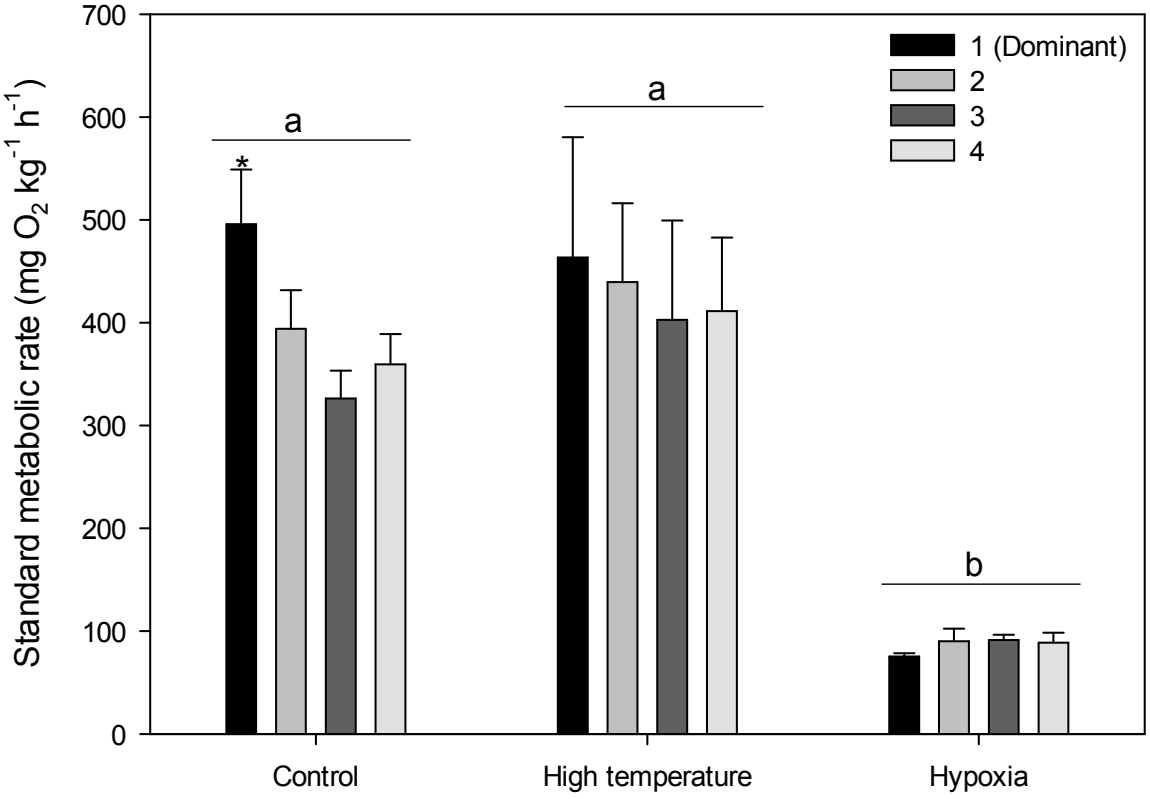
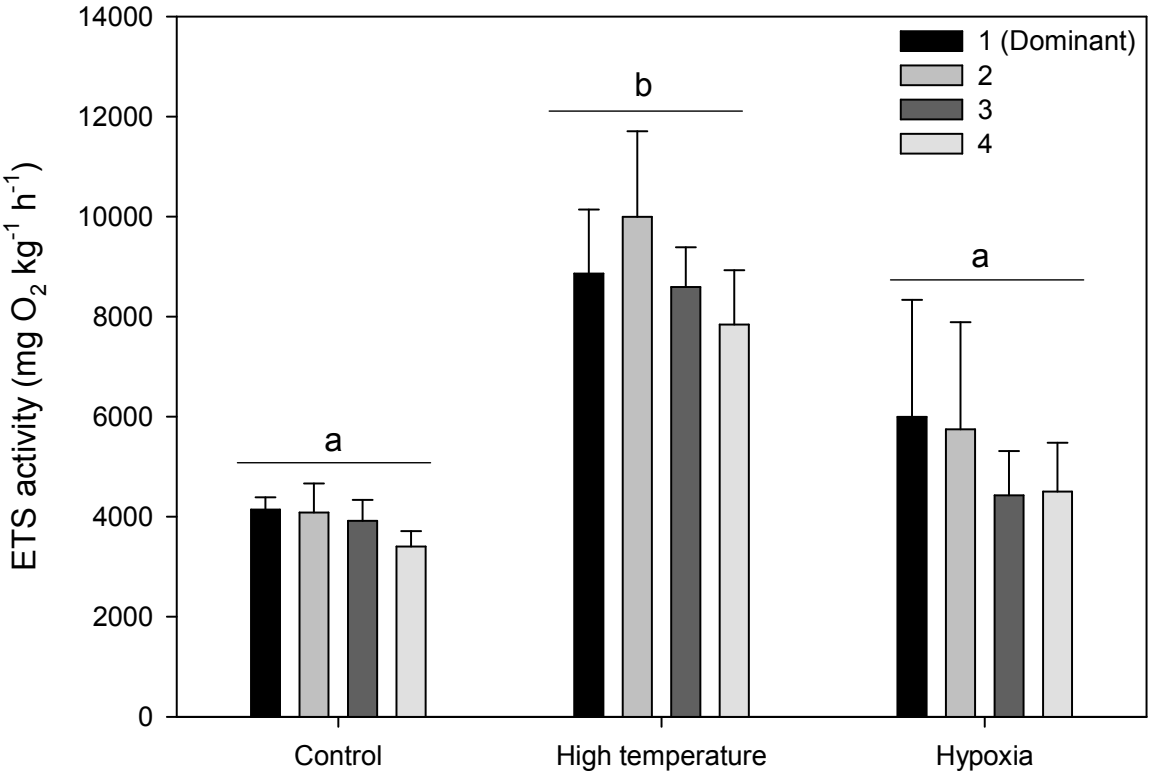
Fig. 6


Standard metabolic rate (mg O_2 kg^{-1} h^{-1})
700
600
500
400
300
200
100
0
1 (Dominant)
2
3
4
a
a
b
*
Control
High temperature
Hypoxia

600 Fig. 7



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Capítulo II

Kochhann, D. & Val, A.L. Social hierarchy and resting metabolic rate in the dwarf cichlid *Apistogramma agassizii*: the role of habitat enrichment. Submetido à Hydrobiologia.

Social hierarchy and resting metabolic rate in the dwarf cichlid *Apistogramma agassizii:* the role of habitat enrichment

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Abstract

Differences in aggressiveness frequently lead to formation of social hierarchy in fish living in groups. Social status acquired is related to changes in physiological parameters, including changes in metabolic rate. Habitat variation can interfere with aggressive behaviour and promote changes in physiological parameters associated with social status. The primary goal of our study was to investigate how differences in habitat complexity can affect the relationship between resting metabolic rate (RMR) and social status in the Amazonian dwarf cichlid *Apistogramma agassizii*. To achieve this goal we compared agonistic interactions (frequency of chasing, tail beating and biting) between pairs of males of this species in aquaria with different habitat enrichment levels (no enrichment, low and high) manipulated by adding shelters. RMR was measured before and after hierarchy definition. Habitat enrichment promotes changes in aggressive behaviour and influences differences in metabolic rate between dominant and subordinate fish. We observed an increase in biting by dominant fish at habitat with high enrichment, which could be related to the increase in territory value. RMR did not predict the dominance as before social interaction it was not different between pairs. We observed an increase in metabolic rate in dominant fish after hierarchy definition. However, this was dependent on habitat enrichment: it occurs only in enriched habitats. We concluded that habitat structure can interfere with behavioural characteristics in social hierarchies and also with physiological consequences of dominance in the dwarf cichlid *Apistogramma agassizii,* as differences in metabolic rate between dominant and subordinate were dependent on habitat structure.

Key words: social hierarchy, fish behaviour, Cichlidae, habitat complexity, habitat enrichment, resting metabolic rate

1 Introduction

The confinement of pairs of individuals in tanks with little or no shelter/refuge, in a situation where one fish will become dominant over the other, the subordinate one is frequently used model to investigate the potential physiological consequences of dominance hierarchies and social interaction in fish is (Sloman et al., 2000b). One of the most relevant consequences of social interactions is the occurrence of changes in metabolic rate. Higher metabolic rate is associated with aggressive behaviour and high aggression rates, and consequently with dominant status. Grantner and Taborsky (1998) observed an increase in metabolic rate in the cichlid *Neolamprologus pulcher* when performing agonistic behaviour, and a higher metabolic rate was also related to the probability of being dominant in the salmonid *Salmo salar* (Metcalfe et al., 1995). That was true also for masu salmon (*Oncorhynchus masou*) where a high correlation between resting metabolic rate and social status was observed (Yamamoto et al., 1998). A high correlation between aggression and metabolic rate was also observed in the cichlid *Oreochromis mossambicus* (Ros et al., 2006). Even though, most studies found a degree of association between social status or aggression and metabolic rate, some of them did not found a clear relationship. Seppanen et al. (2009) studying three populations of Atlantic salmon (*Salmo salar*) did not found any relationship between aggressiveness and metabolic rate, despite differences in metabolic rate between populations. Grobler and Wood (2013) studying the physiology of rainbow trout in social hierarchies found no differences between social status and metabolic rate.

Aggression may also be affected by habitat structural complexity (Schoener, 1987). McCoy and Bell (1991) defined habitat structure as composed of two major factors: complexity and heterogeneity. Complexity can be defined as variation in habitat structure related to absolute abundance of individual structural components (e.g. rocks, trees, leafs). Heterogeneity is the variation in habitat structure due to variation in the relative abundance of different structural components. As stated by Barley and Coleman (2010), an increase in habitat structure may reduce aggression, first, by reducing the frequency with which two individuals encounter each other, and second, by increasing the amount of resources. That was true for pairs of the cichlid *Archocentrus*

nigrofasciatus, when an increase in habitat structure was related to a decrease in aggressive behaviour (Barley & Coleman 2010). Environmental enrichment also decreases aggression in groups of the sparid *Sparus aurata* (Barreto et al., 2011; Batzina et al., 2014; Kelley et al., 2006). Kelley et al. (2006) found that increased habitat structure decreased aggression level in the butterfly splitfin *Ameca splendens*. However, Barreto et al. (2011) found an opposite pattern in Nile tilapia pairs, observing higher aggression rates in higher structurally complex habitat.

However, how changes in habitat structure affect aggressiveness and social status as dominance hierarchies and the relationship between metabolic rate and social status, is much less studied. Increased structural complexity resulted in an increase of growth in *Sparus aurata* reared under different levels of environmental complexity (Batzina et al., 2014), and in rainbow trout (*Oncorhynchus mykiss*) and *Hippoglossus hippoglossus* reared at different substrates (Arndt et al., 2001; Ottesen et al., 2007). Survival in *Heterobranchus longifilis* fry under cage culture conditions also increased with the addition of shelters (Coulibaly et al., 2007).Therefore, the main goal of this study was to investigate how differences in habitat structure can affect the relationship between standard metabolic rate (RMR) and social status in the Amazonian dwarf cichlid *Apistogramma agassizii*.

2 Materials and Methods

2.1 Fish and holding conditions

The dwarf cichlid *Apistogramma agassizii* inhabits clear and black water streams and rivers of Central Amazonia. This species has clear sexual dimorphism. Specimens used in our study were collected in streams close to Tefé Lake (03°29’3561’’S and 64°76’9832’’W), in the municipality of Tefé, Amazonas state, Brazil. After catching they were transferred to plastic bags, partially filled with water and inflated with oxygen, and transported by boat to the Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research in the Amazon (INPA) in Manaus, where the experiments were carried out. In the lab, they were divided into two large stock aquaria

(500L tank), with dead leaves, some trunks and twigs to simulate their natural habitat. They were maintained in continuously aerated tanks, with no water change for at least three weeks before starting the experiment, under a natural photoperiod (12:12 h light: dark). Water temperature, pH and dissolved oxygen were 26.2±0.9°C, 6.6±0.5 and 6.3±0.3 respectively. The animals were fed commercial food pellets with 48% of protein twice a day.

2.2 Fish tagging

Fish were tagged to allow individual recognition using a visible elastomer implant tags (Northwest Marine Technology Inc.) following a standardized protocol recommended by the manufacturer. We used two colours, green and pink along dorsal right and left side with a small bolus of elastomer using a syringe (29gauge, 0.3cc). All tagged fish were then wet weighed (0.71±0.1g) and had the standard length measured (28.92±6.3 mm). Fish were handled without the use of anaesthetics because they can interfere with physiological analysis. Tagging, weighting and measuring take less than 1 min and fish return to normal behaviour one hour after handling. After tagging, fish were held in two aquaria, one for green and another for pink tagged fish to ensure tag retention. Only adult male fish were tagged to control for differences in aggression levels between the sexes (Cole et al., 1980) and to avoid introducing courtship behaviour in the experiment.

2.3 Experimental protocol and procedures

After tagging, two size-matched males were selected from the stock tank. Fish in each pair originated from different stock tanks and were randomly selected to avoid familiarity and previous hierarchical effects. Fish were placed in individual chambers of an intermittent respirometer, and initial oxygen consumption measurements were carried out (section 2.4, Physiological measurements). Fish were then allocated observing size-matched pairs and placed in experimental aquaria (60 cm X 35 cm X 25 cm; 50 L) to determine dominance relationship using behavioural observations. Both fish were introduced into the experimental arena at the same time to avoid effects of

prior residence. Pairs of fish were randomly assigned to experimental arena conditions: control (without any internal structures or objects in the aquaria), level 1 enrichment (composed by one piece of plastic tube of 2 cm diameter and 10 cm long), and level 2 enrichment (composed by two pieces of plastic tube of 2 cm diameter and 10 cm long). The tubes were immediately recognized as shelters by the animals. Fish were given 24 h to acclimate to the environment and to establish a dominance hierarchy. We then returned and monitored the behaviour of the fish for a 30-min period during two consecutive days. During this period, we recorded the number of chases (bursts of increased swimming speed directed to the other fish, that swims to the opposite direction), numbers of tail beats (sudden and heavy undulating movement through the whole body with maximally spread fins) and the number of bites by both fish. Time in the shelter was also measured. All observations occurred between 11:00 h to 15:00 h to control for temporal variation in the activity level of the fish. After the interaction period, each fish of the pair had their metabolic rate measured again.

2.4 RMR measurements

Intermittent-flow respirometry was used to determine the metabolic rate, measured as oxygen consumption. It was calculated by measuring the reduction in oxygen concentration of fully aerated water, flowing past a stationary fish (Steffensen, 1989). We used a computerized apparatus that consists of a recirculation circuit and a 70 mL respiration chamber. The flow in/flush time was as low as possible to achieve replacement of water without disturbing the fish. Feeding was suspended 48h before the measurement to allow the fish to empty their guts. Dissolved oxygen and temperature were automatically monitored and controlled by DAQ-M (Loligo Systems, Tjele, Denmark). Calibration of the oxygen electrode was made using air-saturated water from the header tank to 100% saturation and a solution of sodium sulphite as 0%. Peristaltic pumps were used to flush the chambers with water from the ambient tank during the flush phase. The pumps were off in the measurement phase. The phase time was adjusted to 120 s flush, following by an 180 s wait and 600 s measurement. Thus, the duration of an entire measurement ‘loop’ was 15 min. Oxygen levels in the

chambers were measured using sensor spots, stacked inside of the chambers, and the fiber optic cables were connected to OXY-4 or Witrox 4 (Loligo Systems). Fish were allowed to acclimate to respirometer chamber for 2h before starting the measurements. Metabolic rate was monitored over 3h (12 measurements per fish) and a mean value was calculated. Oxygen consumption rate, MO_2 (mg O_2 $kg^{-1}1$ h^{-1}), was calculated as $MO_2 = -\Delta O_2 Vresp\ B^{-1}$, where O_2 is the rate of change in oxygen tension (kPa h^{-1}), Vresp is the volume of the respirometer, and B is the mass of the individual (kg).

2.5 Statistical analyses

All values are presented as mean (± Standard error of the mean - SEM). To compare the effects of the levels of habitat structure on aggression levels between treatments and social position, analyzed by me as the total number of chases, the total numbers of tail beat, the number of bites, and the whole aggressiveness (calculated as the sum of all aggressive acts) in each fish between the treatments. Fish that has the higher number of aggressive acts was called dominant, and the other, subordinate. Wherever necessary, the data were log-transformed in order to obtain a normal distribution and homogeneity of variances, tested with Levene's and Kolmogorov's tests respectively. As there was no difference in aggressiveness between 24h and 48h observation time, data were pooled for subsequent analyses. We used Two-way ANOVA to compare aggressive interactions, with enrichment conditions and social status as independent variables, with post hoc Scheffé's test. A two-way ANOVA for repeated measurements was employed to compare RMR before and after social interaction, with enrichment conditions and social status as independent variables and RMRs as repeated measurements, again using post hoc Scheffé's test. Differences in metabolic rate between social status after social interaction was tested by paired Student's t-tests. Statistical differences were considered significant at $P \leq 0.05$.

3 Results

Social hierarchy was clearly defined by agonistic interactions (Figure 1). The two-way ANOVA revealed an effect of social hierarchy ($F = 55.37$; $P < 0.001$), but no effect of enrichment condition ($F = 0.66$; $P = 0.51$) or interaction effect ($F = 0.66$; $P = 0.51$) on chasing (Fig. 1A). Similar results were observed for tail beating (Fig. 1B; social hierarchy: $F = 23.61$; $P < 0.001$; enrichment condition: $F = 0.24$; $P = 0.78$; interaction: $F = 0.26$; $P = 0.77$) and total agonistic acts (Fig. 1D; social hierarchy: $F = 63.49$; $P < 0.001$; enrichment condition: $F = 1.05$; $P = 0.35$; interaction: $F = 1.07$; $P = 0.34$). Biting was the only social interaction with changes in frequency related to habitat enrichment, with significant effects of social hierarchy ($F = 25.70$; $P < 0.001$), of enrichment condition ($F = 3.55$; $P = 0.03$) and an interaction effect ($F = 3.55$; $P = 0.03$) (Fig. 1B).

Shelter use was also not influenced by enrichment conditions. The two-way ANOVA revealed an effect of social hierarchy ($F = 16.29$; $P < 0.001$), but no effect of enrichment condition ($F = 2.68$; $P = 0.11$) or interaction effect ($F = 1.83$; $P = 0.18$) on shelter use (Fig. 2). An important observation was that in the enriched condition 2, dominant fish monopolized both shelters.

Repeated measures two-way ANOVA revealed a significant effect of social hierarchy ($F = 11.01$; $P < 0.001$), but no effect of enrichment condition ($F = 1.30$; $P = 0.27$) and a marginally, but not significant, interaction effect ($F = 2.46$; $P = 0.051$) on changes in RMR (Fig. 3). When compared independently, a difference in RMR between dominant and subordinate fish was only observed after social interaction in fish at the low enrichment condition (Fig. 4).

4 Discussion

In nature, competition by limited resources is frequently expressed as aggressive disputes (Ridley, 1995). Resources include, among others, food, reproductive partners and shelters. The addition of structures that can be used as shelters, with a consequent increase in habitat complexity and heterogeneity, are frequently used to improve welfare in captive animals, including in fish (e.g. Salvanes and Braithwaite, 2005, 2006). However, an increase in habitat structure can also increase motivation to win a contest,

by increasing the value of the resource space, in this case. This increase in motivation can reflect in an increase in aggressiveness (Kadry and Barreto, 2010). In our study, most of the aggressive parameters did not change with differences in habitat enrichment. However, biting, that can be considered the most energetically costly agonistic interaction, due to the possibility to cause physical damage, increased in habitats with high habitat enrichment. This result disagrees with the studies of Barley and Coleman (2010) and Kadry and Barreto (2010), both with cichlid fishes. These authors observed a reduction of fish aggressiveness under increased habitat complexity.

For the dwarf cichlid *Apistogramma agassizii*, a territorial small fish that lives in shallow and transparent waters, a shelter will help to protect from predation from bigger fishes or even from aerial predators. A potential increase in mate acquisition could also be responsible for the increased value of environmental resources (Nijman and Heuts, 2000). Barreto et al. (2011) also observed an increase in aggressive acts in Nile tilapia in environments with higher habitat enrichment, agreeing with game theory predictions. Our results are in line with game theory, a model that predicts that costs of fighting should increase with the resource value (Arnott and Elwood, 2008; Bishop et al., 1978). So, as fish recognize an increase in resource value (in this case, an increase in shelter numbers) they may invest more energy to defend this resource.

Our results pointed out that resting metabolic rate (RMR) before the definition of social hierarchy in the experiments did not predict the dominance. In contrast to our expectations, RMR was not higher in fish that become dominant. In their review, Biro and Stamps (2010) stated that a higher RMR should be required to sustain higher metabolic costs. So we expected that individuals who became dominant would have higher metabolic rates, as observed by Metcalfe et al. (1995) for *Salmo salar*. These authors observed that the higher the metabolic rate, the higher was the probability of a given fish to become dominant, concluding that a high metabolic rate is rather a cause than a consequence of dominance status. That contrasts with our data for *Apistogramma agassizii*. However, after the social hierarchy definition we observed changes in RMR. The effect of social hierarchy on the RMR of the dwarf cichlid depended on social status. The period of social interaction resulted in subordinates

exhibiting a decrease in RMR while the RMR of dominants increased. Higher metabolic rate is frequently associated with a dominant status (Grantner and Taborsky, 1998; Metcalfe et al., 1995; Vollestad and Quinn, 2003; Yamamoto et al., 1998) and our results corroborate this study. The increase in RMR are related to the metabolic costs of agonistic behaviour that, when performed, are related to an increase in oxygen consumption (Castro et al., 2006). However, higher metabolic rates are a key characteristic for higher growth rates (Hoogenboom et al., 2012). These authors observed that territorial aggressive behaviour only promoted growth when food was predictable, and only for individuals that had high metabolic rates.

Overall, habitat enrichment did not influence RMR. Even though, we can see an increasing tendency (not significant) in dominants and a decrease in subordinates when habitat enrichment increases. However, when we compare metabolic rate of dominant and subordinate fish in each enrichment condition we observe an increase in RMR in dominant in the enriched conditions. Higher metabolic rate, instead of being a cost, as it would be expected, seems to be the way the dominant fish achieve higher growth rate (Hoogenboom et al., 2012; Millidine et al., 2009b). So, a potential benefit of dominant position in growth rate is expected only when dominant fish have access to shelter. Higher growth rate can affect all biological parameters related to fitness, promoting higher access to food and mates.

Altogether, our results support the hypothesis that the dominant fish have higher RMR when compared to subordinate, as observed before (Grantner and Taborsky, 1998; Metcalfe et al., 1995; Vollestad and Quinn, 2003; Yamamoto et al., 1998). However, this was dependent on habitat structure in the present study. Only enriched habitats can sustain dominants with higher metabolic rates compared to subordinates. Despite not having observed an increase in overall aggressiveness in fish at the enriched environment, we observed an increase in biting. This increase in the most costly aggressive behaviour, biting, which is in line with game theory predictions that higher resource values can increase the amount of energy to defend it. Long-term studies evaluating the effect of habitat structure on social behaviour and growth of the dwarf cichlid *Apistogramma agassizii* should be carried out to clarify these relationships.

5 Acknowledgments

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373 Figure captions

374 **Figure 1.** The effects of environmental enrichment on categories of aggressive behavior
375 in *Apistogramma agassizii*: (A) chasing, (B) biting, (C) tail beat, and (D) the sum of all of
376 them. The data were compared by Two-Way ANOVA followed by Scheffé's test (n= 8
377 pairs for each condition). * denotes a significant (P< 0.05) statistical difference between
378 dominant and subordinate fish within an enrichment condition and the letters a and b
379 denote a significant (P< 0.05) statistical difference at pair level between enrichment
380 conditions.

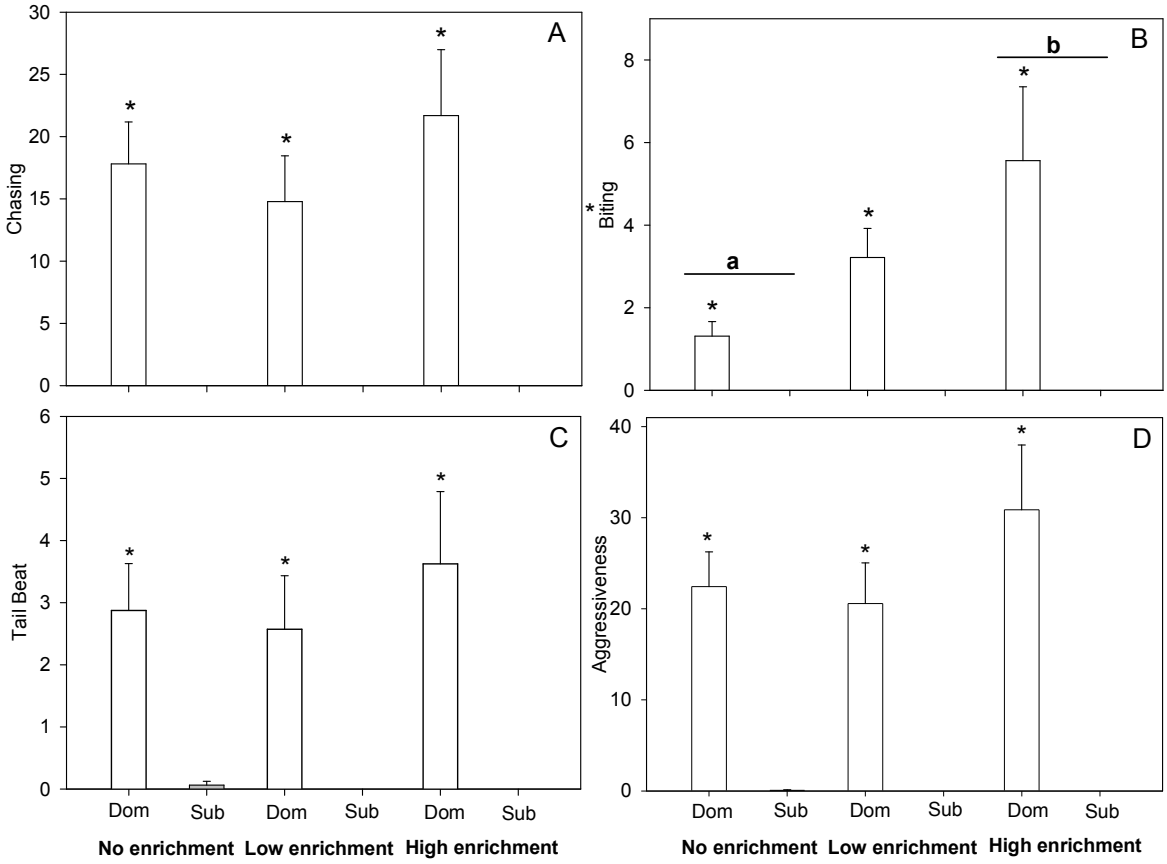
381 **Figure 2.** The effects of environmental enrichment on shelter use in *Apistogramma*
382 *agassizii*. The data were compared by Two-Way ANOVA followed by Scheffé's test (n=
383 8 pairs for each condition). *denotes a significant (P< 0.05) statistical difference
384 between dominant and subordinate fish within an enrichment condition.

385 **Figure 3.** The effect of social position on changes of resting metabolic rate (RMR) in
386 *Apistogramma agassizii* before and after social interaction.

387 **Figure 4.** The effects of environmental enrichment on resting metabolic rate (RMR) in
388 *Apistogramma agassizii*. The data were compared by Two-Way ANOVA followed by
389 Scheffé's test (n= 8 pairs for each condition). * denotes a significant (P< 0.05) statistical
390 difference between dominant and subordinate fish within an enrichment condition.

391

392 Fig. 1



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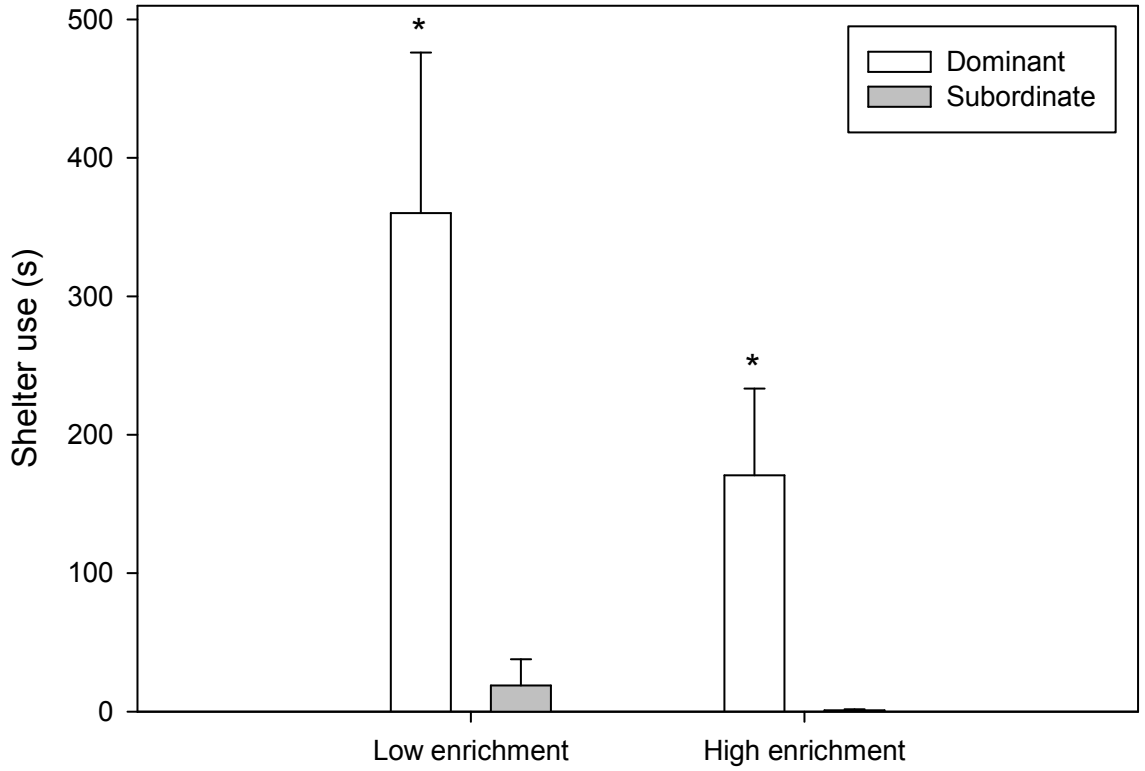
396 Fig. 2



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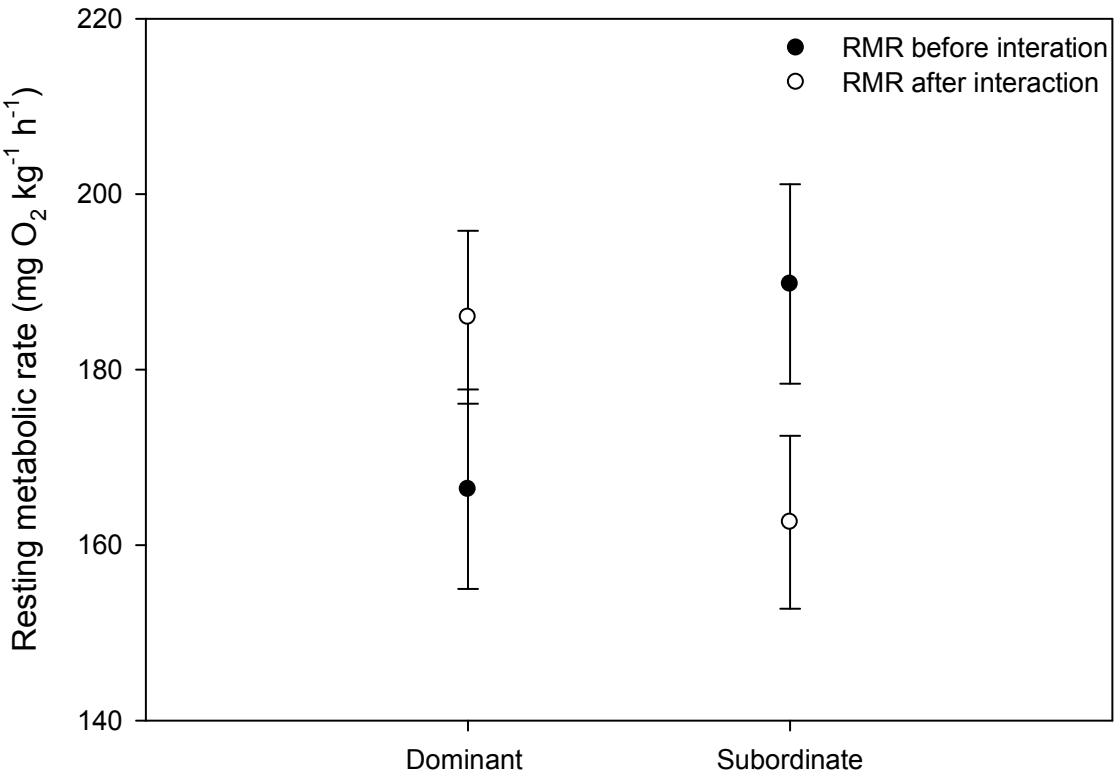
399 Fig. 3



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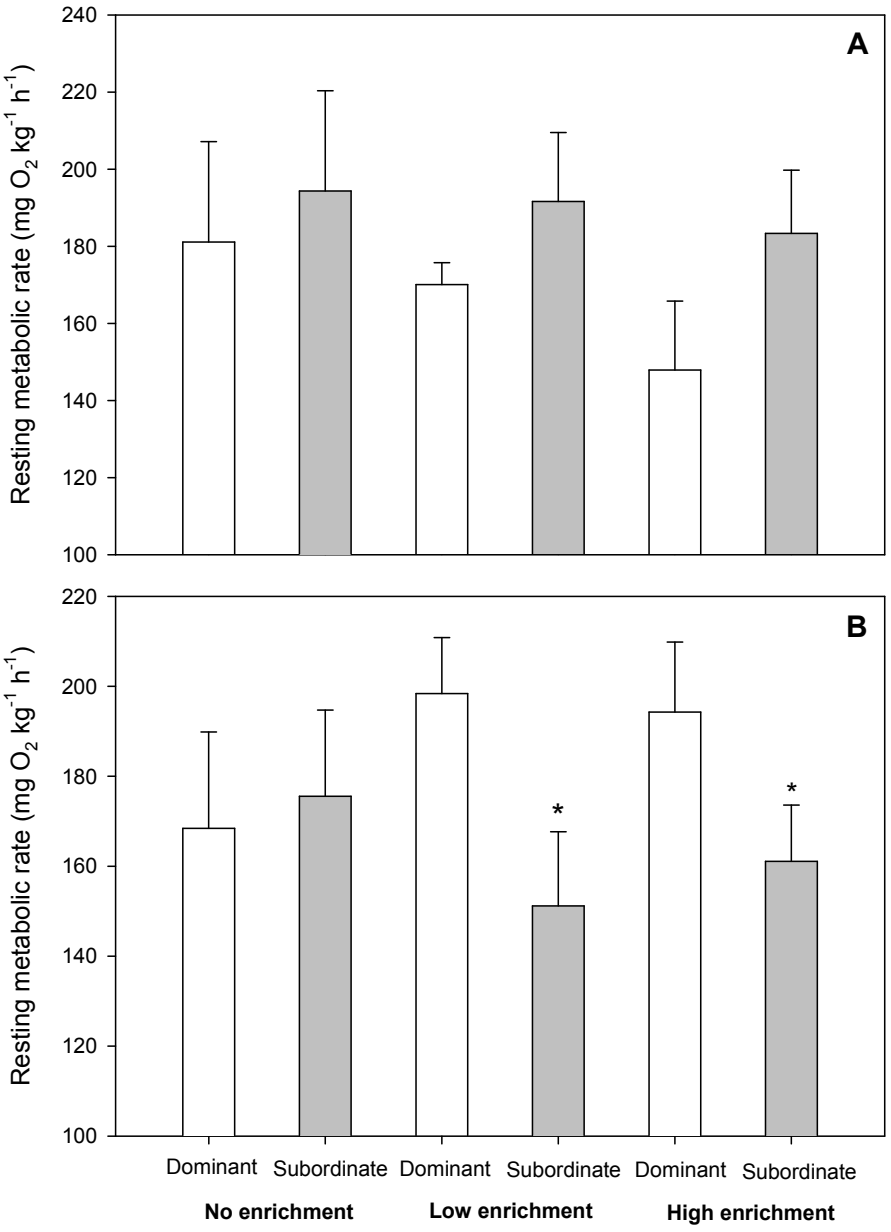
402 Fig. 4


A
B
Resting metabolic rate (mg O_2 kg^{-1} h^{-1})
240
220
200
180
160
140
120
100
*
*
Dominant
Subordinate
Dominant
Subordinate
Dominant
Subordinate
No enrichment
Low enrichment
High enrichment

403

Capítulo III

Kochhann, D. & Val, A.L. Metabolic correlates of aggressiveness in two populations of the Amazonian dwarf cichlid *Apistogramma hippolytae*.

Metabolic correlates of aggressiveness in two population of the Amazonian dwarf cichlid *Apistogramma hippolytae*

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Abstract

Group-living animals frequently form social hierarchies, and aggressive behaviour is a key characteristic of dominant animals. Dominant individuals typically have higher metabolic rates to maintain their aggressiveness. The primary goal of our study was to investigate the relationship between metabolic rate and social position of the Amazonian dwarf cichlid *Apistogramma hippolytae* in their natural environment. To achieve this goal we analysed metabolic correlates of social position in two populations *A. hippolytae*. We were able to identify stable social hierarchies in the two populations. We found differences in aggressiveness and resting metabolic rate between animals from the two studied populations (Amanã and Dimona Farm). Fish from Dimona had higher aggressiveness and feeding rates, whereas fish from Amanã have higher resting metabolic rate. Dominant fish had metabolic advantages in both studied sites; however, in Amanã the most subordinate fish had metabolic scope as high as dominant. The differences observed in physiology and behaviour between the populations of the two studied sites could be due to habitat variation. Altogether, our results support the hypothesis that the dominant fish have metabolic differences when compared with subordinate fish. However, differences in habitat and among populations can promote changes in physiological correlates of dominance in populations of *Apistogramma hippolytae*.

Key words: aggression, hierarchy, aerobic metabolism, dwarf cichlid, metabolic rate

1 Introduction

In species living in social groups, aggression among individuals to gain access to limiting resources can lead to the formation of stable social hierarchies (Whiteman and Côté, 2004). Establishment of hierarchies is accomplished through agonistic behaviour, where fish use aggression to compete for a limiting resource and attain higher social position (Grobler and Wood, 2013). Maintenance of social hierarchies is thought to be advantageous for fish independent of social position. It is known that the reduction of aggressive behaviour can cause serious damage and be energetically costly (Hesse and Thünken, 2014; Hick et al., 2014).

However, if fish fight to be dominant, we expect individual benefits for the animals reaching dominant position within a group. Higher metabolic rate is commonly associated with high aggression rates, and consequently with dominant status (Grantner and Taborsky, 1998; Metcalfe et al., 1995; Vollestad and Quinn, 2003; Yamamoto et al., 1998). Higher metabolic rate, instead of being a cost, as it would be expected, seems to be the way dominant fish achieve higher growth rate, even though this advantage is dependent of habitat complexity (Hoogenboom et al., 2013; Millidine et al., 2009a). However, as far as we know, the relationship between metabolic rate and social position was never evaluated under field conditions. Investigating physiological correlates of dominance in *Salmo trutta* natural populations, Sloman et al. (2008) observed that they do occur, but may differ from those found in the laboratory. For example, in natural populations of *Salmo trutta,* no relationship between social position and growth rate was observed, and dominant fish had higher plasma cortisol concentrations (Sloman et al., 2008).

Measurement of oxygen consumption is a good indicator for the intensity of metabolism under particular conditions. However, how much of the measured oxygen consumption is related to the metabolic capacity remains to be elucidated. The activity of electron transport system (ETS) has been measured to estimate the potential metabolic activity, i.e. the oxygen consumption that would occur if all enzymes function at maximum rates (Muskó et al., 1995), what can be used as a measurement of metabolic capacity. Theoretically a high ETS/RMR ratio is desirable, allowing for a large aerobic scope, and can be used as an effective tool for assessing the general metabolic condition of aquatic animals (Lukančič et al., 2010).

Therefore, the objective of our study is to analyse the relationship between these metabolic parameters and social position of fish in their natural environment. We choose a species of the cichlid genus *Apistogramma* for this study. *Apistogramma* includes small shelter-breeding fish that live in the leaf litter of small rivers and brooks (Römer and Beisenherz, 1996).The studied species *A. hippolytae* inhabits clear and black water streams and rivers of Central Amazonia, and some behavioural studies were previously carried out for this fish (see Rodrigues et al., 2009; Rodrigues et al., 2012). We hypothesized that dominant fish will have higher routine metabolic rate, but also higher ETS activity what will lead to higher ETS/RMR ratios.

2 Methods

2.1 Study areas

We studied the behaviour of *Apistogramma hippolytae* at two sites. The first study site was an impounded stretch of a forest stream at Dimona Farm research station (2°20′25.5114″S/60°6′5.7594″W) in the in the area of the Biological Dynamics of Forest Fragments Project (BDFFP), during September of 2013. The area is located 70 km north of Manaus, Brazil, and the studied stream is part of Cuieiras River basin, a tributary to Rio Negro. The pond has approximately 40m long, 14 m wide and 0.5m deep. The bottom consisted mainly of dead leaves with trunks and twigs, all covered by a layer of fine sediments in which the studied species feed. The pond is surrounded by primary and secondary tropical rain forest. Physicochemical characteristics of water during the studied period ranged as follow: dissolved oxygen 3.19 - 3.69 mg/L, pH 4.7 - 4.9 and temperature 24.6 - 27.9°C. The water is clear, allowing underwater and supra-aquatic observations. Additional information on this collecting site has been reported by Rodrigues et al. (2009; 2012). The second study site was a streamlet at the Biological Reserve Amanã (2°28′53.283″S/64°37′47.792″W). The area is located 650 km west of Manaus, Brazil, between Japurá and Negro river basins. For experimental purposes we delimited an area of the streamlet of approximately 14m long, 4m wide, and 0.5m deep, using two fine-meshed nets. The bottom consisted of dead leaves, sand and some trunks, with some fine sediment, but lesser than observed in Dimona area. The streamlet is surrounded by primary tropical rain forest. Physicochemical characteristics of water during the studied period ranged as follow: dissolved oxygen 3.89 - 4.56 mg/L;

pH 4.5 - 4.8 and temperature from 24.1- 25.8°C. The water was clear, allowing underwater and supra-aquatic observations.

2.2 Fish sampling and tagging

Fish were captured using a hand net and then tagged to allow individual recognition using visible elastomer implant tags (Northwest Marine Technology Inc.) following a standardized protocol. We used a unique combination of seven coloured tags and four body locations along dorsal fin side, two at the left and two at the right side, injected subcutaneously using a syringe (29 gauge, 1cc). All tagged fish were then wet weighed (mean±SD Amanã: 0.76±0.28g, Dimona: 0.86±0.81g) and had the standard length measured (mean±SD Amanã: 28.49±3.27g, Dimona: 27.55±7.51g). After tagging, all fish were held in the laboratory for two days, to allow for recovery and to ensure tag retention, before being released to observational field site. At Dimona sampling site, fish were captured in the whole pond and released all at same the point, in Amanã streamlet, fish from the vicinity of the observational point was also captured and they were all released at the same point.

2.3 Behavioural data collection

Feeding and aggressive behaviour data were acquired from direct observations in the natural habitat. Observations were made during one week after release. In Dimona, we conducted 40h of observation, and in Amanã 24h, both underwater (snorkelling) and supra-aquatic (in places where the depth was less than 0.5m). Focal animal observations (Altman, 1974) were made for each fish between 7 AM and 4 PM as this was the period of higher activity of the fish (personal observation). The same

observer (DK) made all field observations. The observer remained stationary in a distance of ±1m from the observed individual and waited at least 15 min to begin observations. The distance was enough to identify tagging and also to avoid any fish disturbance. During observation, all foraging and aggressive events were recorded. Foraging intensity was estimated as the number of feeding bites recorded at the substratum at least three times during different periods of the day. Group hierarchy was defined through aggressive events in a single observation of each group. Hierarchy was defined examining all dyadic interactions inside each group and groups were defined as the smaller social groups to which the individual fish belongs. Groups of fishes were collected after three consecutive observations for physiological data collection. The average number of feeding bites and aggressive events per minute per individual were determined and used as behavioural variables.

2.4 Physiological data collection

After estimation of the hierarchy, fish were recaptured with hand nets and transferred to individual aquaria (1L) to recover from recapture stress for 24h. Routine metabolic rate (RMR) was measured in the field. Fish were starved during this time. RMR was measured as oxygen consumption and was determined after the recovery period using the closed bottle method (Lampert, 1984a). For this measurement, fish was gently transferred to an individual glass bottle (500mL) filled with aerated water from the streamlet where the fish was collected. Each bottle had a sensor allowing measurement of oxygen from the outside, without disturbing the fish. All bottles were sealed with Parafilm® and kept at averaged temperature of the water at the study point (27±0.1°C at Dimona and 25±0.1°C at Amanã). The experimental bottles received one

154 animal per bottle, while three bottles served as controls. The first measurement was
155 made 20 min after the beginning of incubation to allow animals to recover from handling
156 stress, and lasted for three hours. Measurements were taken always at night, between
157 7 and 10:30 PM to avoid diurnal metabolic fluctuations. Oxygen in the experimental and
158 control bottles were measured with a 4-channel fiber-optic oxygen meter (PreSens
159 OXY-4, Presens GmbH, Egensburg, Germany). Measurement of oxygen was made by
160 touching the sensor from the outside, so only one channel were used to measure all
161 bottles. The difference between the concentrations of dissolved oxygen of each
162 experimental bottle at the start and the end of incubation (± 1.0mg L O_2), minus the
163 mean value of control bottles, was taken as the amount of oxygen consumed by
164 animals. After RMR measurements, fish were identified, weighed, measured, rapidly
165 decapitated and immediately transferred to liquid nitrogen container for measurements
166 of electron transport system activity (ETS). ETS were measured in the laboratory using
167 the method proposed by Packard (1971) and improved by G.-Tóth (1993). The solutions
168 were prepared 24h before experiments and maintained on ice to avoid substrate
169 decomposition and bacterial contamination. The whole animal was homogenised with
170 liquid nitrogen using a mortar and a pestle. The pre-weighed homogenised material (30-
171 60 mg wet mass) was then sonicated using a Sonifier (VWR Scientific, model 450) in 4
172 mL of ice-cold homogenisation buffer (0.1M sodium phosphate buffer pH = 8.4; 75 µM
173 $MgSO_4$; 0.15%(w/v) polyvinyl pyrrolidone; 0.2%(v/v) Triton-X-100) for 20 s and
174 centrifuged for 4 min at 0°C at 8500*g* using a Eppendorf centrifuge 5430R. Three 0.5-
175 mL samples from each homogenate were incubated in 1.5 mL substrate solution (0.1M
176 sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2%(v/v) Triton-

X-100) with 0.5 mL 2.5 mM 2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyl tetrazolium chloride (INT) solution for 20 min at the same temperature that RMR was measured (27°C at Dimona and 25°C at Amanã). The reaction was stopped by adding 0.5 mL of 1:1 formalin:H_3PO_4. Blanks (1.5 mL substrate solution and 0.5 mL INT solution) were incubated and stopped as for the samples; 0.5 mL of homogenate was added after the reaction was stopped. Formazan production was determined spectrophotometrically from the absorbance of the sample at 490 nm against the blank, within 10 min of stopping the reaction, using a spectrophotometer Spectramax Plus 384 Molecular Device. ETS activity was measured as the rate of tetrazolium dye reduction to formazan, and converted to oxygen equivalent consumed per mg of wet mass per hour ($\mu LO_2 mgWW^{-1}h^{-1}$), as described by Kenner & Ahmed (1975).

ETS activity/respiration ratios (ETS/RMR) were calculated from the ratio of maximum oxygen consumption (ETS), measured as in vitro enzymatic rates, to the rate of respiration (RMR), determined in vivo per gram of wet mass (Owens and King, 1975).

To validate RMR measured in the field, individuals of a close related species, *Apistogramma agassizi* were collected in streams close to Tefé Lake (03°29'3561''S and 64°76'9832''W). After catching they were transferred to plastic bags, partially filled with water and inflated with oxygen, and transported by boat to the Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research in the Amazon (INPA), Manaus. In the lab, they were maintained in a large stock aquaria (500L tank), with dead leaves, trunks and twigs to simulate their natural habitat. They were maintained in continuously aerated tanks, with no water change for at least three weeks before starting the experiment, under a natural photoperiod (12:12 h light:dark).

200 Water temperature, pH and dissolved oxygen were 26.2±0.9°C, 6.6±0.5 and 6.3±0.3 O_2
201 mgL^{-1} respectively. The animals were fed commercial fish food pellets with 48% of
202 protein twice a day. Intermittent-flow and closed bottle respirometry were used to
203 determine the metabolic rate of eight fish, in order to compare if the low time of
204 acclimation to closed bottle could increase metabolic rate due to handle stress. To
205 measure metabolic rate by intermittent-flow respirometry we used a computerized
206 apparatus which consists of a recirculation circuit and a 70 mL respiration chamber. The
207 flow in/flush time was as low as possible to achieve replacement of water without
208 disturbing the fish. Feeding was suspended 24h before the measurement to allow the
209 fish to empty their guts. Dissolved oxygen and temperature were automatically
210 monitored and controlled by DAQ-M (Loligo Systems, Tjele, Denmark). Calibration of
211 oxygen electrode was made using air-saturated water from the header tank as 100%
212 saturation and a solution of sodium sulphite as 0%. Peristaltic pumps were used to flush
213 the chambers with water from the ambient tank during the flush phase. The pumps were
214 off during measurement phase. The phase time were adjusted to 120 s flush, following
215 by a 180 s wait and 600 s measurement. Thus, the duration of an entire measurement
216 'loop' was 15 min. Oxygen levels in the chambers were measured using sensor spots,
217 stacked inside of the chambers, and the fiber optic cables were connected to OXY-4 or
218 Witrox 4 (Loligo Systems). Fish were allowed to acclimate to respirometer chamber for
219 3h before starting the measurements. Metabolic rate was monitored over 3h (12
220 measurements per fish) and a mean value was calculated. Oxygen consumption rate,
221 MO_2 (mg O_2 $kg^{-1}1$ h^{-1}), was calculated as $MO_2 = -\Delta O_2 Vresp\ B^{-1}$, where O_2 is the rate of
222 change in oxygen tension (kPa h^{-1}), Vresp is the volume of the respirometer, and B is

223 the mass of the experimental animal (kg). After the intermittent –flow respirometry
224 measurement, fish were gently transferred to the same glass bottles that were used in
225 the field for closed bottles respirometry measurements. Procedure was exactly the
226 same as used in the field. The result was compared using the t-test. Mean RMR for
227 intermittent-flow respirometry was 186±14.8 mg O_2 $kg^{-1}1$ h^{-1} whereas for closed bottle
228 was 200.0±10.1 mg O_2 $kg^{-1}1$ h^{-1}. There was no difference in the measurement ($p=0.46$;
229 $t=-0.745$ with 14 degrees of freedom).

230 2.5 Statistical analyses

231 Statistical analyses were conducted using SigmaStat 3.5 (Systat Software Inc.
232 2006) and Statistica 7.0 (StatSoft Inc. 2004). Data normality was analyzed by Shapiro
233 Wilk's test, and parametric and non-parametric statistical tests applied accordingly. We
234 used t-test to compare differences in behavioural and physiological parameters between
235 sites and for the validation of RMR measurements. We used simple Spearman
236 correlations to evaluate relationships between aggression rates and behaviour, and
237 agression rates and physiological parameters. Weight-specific values were used to
238 standardize oxygen consumed per gram of body mass. As we found allometric scaling
239 between metabolic rate and mass, RMR was regressed to mass to calculate residuals
240 (observed-predicted) for each individual. As ETS/RMR ratio is dependent of body size
241 (Cammen et al., 1990; Simčič et al., 2012) we also performed residual analysis for this
242 parameter. Probability levels have been reported throughout, and $p<0.05$ was
243 considered significant.

244 2.6 Ethical note

245 This study was approved by the institutional animal care committee of the
246 National Institute for Research in the Amazon, Manaus (Permit number 012/2012). Fish
247 were marked with visible implant elastomer in the dorsal flank. Fish were handles
248 without the use of anaesthetics because they can interfere with physiological analysis.
249 Tagging and body measurement take less than 30s and fish perform normal behaviour
250 less than 10 min after tagging. After analysis, fish were rapid decapitated, an approved
251 method of euthanasia in fishes.

252

253 **3 Results**

254 We collected, tagged and released, respectively, 189 and 161 individuals of
255 *Apistogramma hippolytae*, in Dimona and Amanã sites respectively. After observation,
256 we recaptured 28 animals in Dimona. These animals were originally from seven groups,
257 one group with five individuals, five groups with four individuals each, and one group
258 with three individuals. In Amanã we recaptured 23 individuals from six groups, one
259 group with three individuals and five with four individuals each. Only animals that were
260 recaptured were used in the analysis, allowing us to compare physiological parameters
261 directly with behavior.

262 We found significant differences in behavior and physiological parameters
263 between the two analyzed sites. Aggressiveness and feeding rate were higher in
264 Dimona site (aggressiveness: $P = 0.041$; feeding rate: $P < 0.001$; Fig 1 A and B).
265 However, respiration rate was higher in Amanã, and ETS slightly, but not statistically
266 different, higher in Amanã comparing to Dimona (respiration rate: $P < 0.001$; electron

267 transport system activity: *P* = 0.052; Fig 1 C and D). The difference of ETS/R between
268 the two sites was also not insignificant (*P* = 0.855, Fig 1 E).

269 The hierarchies appeared to be stable at both sites. There was a significant
270 correlation between aggressiveness and rank position within each analysed site
271 (Dimona *rs* = -0.95, *n* = 28, *P* < 0.001, Amanã *rs* = -0.963, *n* = 23, *P* < 0.001). Feeding
272 showed a positive and significant correlation with aggressiveness in both studied sites
273 (Amanã *rs* = 0.658, *n* = 23, *P* < 0.001; Dimona *rs* = 0.393, *n* = 28, *P* = 0.03).

274 We did not find a relationship between ETS activity and fish wet mass in both
275 sites (Amanã *rs* = 0.140, *n* = 23, *P* = 0.51; Dimona *rs* = 0.248, *n* = 28, *P* = 0.20, Table
276 2). However, there was a positive and significant correlation between ETS activity and
277 aggressiveness in Dimona site (*rs* = 0.78, *n* = 28, *P* < 0.001). This relationship was not
278 observed for animals in Amanã (*rs* = 0.334, *n* = 23, *P* = 0.12). We did not observe a
279 relationship between aggressiveness and RMR in any site (Amanã *rs* = -0.217, *n* = 23,
280 *P* = 0.32; Dimona *rs* = 0.238, *n* = 28, *P* = 0.22, Table 1), but, as expected, there was a
281 significant negative correlation between RMR and fish wet mass (Amanã *rs* = -0.551, *n*
282 = 23, *P* = 0.006; Dimona *rs* = -0.763, *n* = 28, *P* < 0.001, Table 2). So, we regressed
283 RMR to mass to calculate residuals (observed-predicted) for each individual and found
284 no relationship between RMR and rank position (Figure 2 A and B).

285 In addition, a significant positive relationship between aggressiveness and
286 ETS/RMR rate was observed only at Dimona site (Dimona *rs* = 0.74, *n* = 28, *P* <0.001;
287 Amanã *rs* = 0.398, *n* = 23, *P* = 0.06, Table 1). Surprisingly, there was also a significant
288 correlation between ETS/RMR rates and wet mass only at Dimona site (Dimona *rs* =

289 0.633, *n* = 28, *P* <0.001; Amanã *rs* = 0.372, *n* = 23, *P* = 0.08, Table 2). Regressed rates
290 ETS/RMR to mass to calculate residuals (observed-predicted) for each individual in
291 both sites showed that fish from a dominant position had a tendency to have higher
292 ETS/RMR rates than expected at Dimona site (Figure 3A). In Amanã site, we also
293 observed that dominant fish tend to have higher ETS/RMR. However, this also occurred
294 for fish of lower social rank position (Figure 3B).

295

296 **4. Discussion**

297 Linear hierarchies of dominance were recognized among individuals of
298 *Apistogramma hippolytae* inhabiting both Dimona and Amanã sites. However, we found
299 strong behavioural differences between both sites. Feeding and aggressiveness rates
300 were both higher at Dimona site. Difference in aggressiveness between populations or
301 strains was also found in previous studies. Kagawa (2013) examined two wild
302 populations of two closely related species, *Oryzias latipes* and *O. sakaizumii* (Japanese
303 medaka, Adrianichthyidae) in the laboratory and found higher aggressiveness by
304 dominants individuals of groups from the South when compared to groups from
305 Northern population. Differences in aggressiveness were also observed in two strains of
306 rainbow trout (Schjolden et al., 2005). As both studies were carried out under
307 standardized laboratory conditions, those differences are arguably inherent to the
308 individuals. In our study, we cannot say that differences observed in aggressiveness
309 were related to variation among populations as we were unable to disentangle genetic
310 and environmental factors.

311 Although not measured, water flow was higher in Amanã site. Sneddon,
312 Hawkesworth, Braithwaite, & Yerbury (2006) simulating an increase of turbulence in
313 aquaria at the lab did not find any difference in aggressive behaviour in dominance
314 hierarchies of three-spined sticklebacks, *Gasterosteus aculeatus*. Costs of station-
315 holding maintenance under high water flow, particularly for long time periods, could lead
316 to a decrease in aggressive acts, as both are costly (Grantner and Taborsky, 1998).

317 Feeding rate was lower in fish from Amanã population. This is a contrasting
318 result as fish from Amanã also have higher metabolic rates when compared to fish from
319 Dimona. Fish with higher metabolic rate, have a higher cost-of-living and increased
320 metabolic demands (Metcalfe et al., 1995) that need more food to be sustained. As the
321 analysed *Apistogramma* species feed on the substratum, eating plant debris together
322 with small invertebrates (Carvalho, 2008), differences in environmental characteristics
323 of both sites (i.e. food of higher quality at Dimona) may explain the reduced foraging
324 rate even with a higher metabolic rate. Electron transport activity was also higher in fish
325 from the Amanã site. This was expected as this is a measurement of aerobic capacity
326 (Muskó et al., 1995) that typically correlates with environmental oxygen, which was
327 higher in Amanã when compared to Dimona site. The quotient ETS/RMR is an
328 indication of the metabolic potential (Martinez, 1992), i.e. an indication of how much of
329 the whole metabolic capacity of the fish is being used at that specific moment. As both
330 ETS and RMR were higher in fishes of Amanã, we did not find a significant difference
331 between sites regards ETS/RMR, suggesting that fish from both sites have equivalent
332 metabolic scope.

Distribution of agonistic behaviour between individuals has been accepted as the most useful criterion for measuring dominance, as this is a characteristic of all interactions between individuals in a group (Sneddon et al., 2006). Our study has shown that aggressiveness rate was highly correlated with rank position in both studied sites. Intraspecific competition is often displayed as dominance hierarchies whereby aggressive individuals use a greater proportion of a resource, as mates, shelter, and food, than smaller or subordinate individuals (Webster and Hixon, 2000). In our study, feeding rate was significantly related to aggressiveness in both studied sites. Although social dominance in fish has just occasionally been examined in the wild, some studies also found a positive relationship between rank position and feeding rate (Nakano, 1995; Webster and Hixon, 2000). Forrester (1991) did not find any relationship between feeding rate and social position, but high-ranked fish consumed significantly larger prey than low-ranked fish. Taken together, these results support the hypothesis of Whiteman and Côté (2004) that intraspecific competition for resources creates dominance hierarchies and provides support for the role of individual attributes in the formation and maintenance of such hierarchies. So, competition for food patches reflects in the aggressiveness and creates and maintains the dominance hierarchy, as observed for the analysed species. Metabolic rate is often analysed in dominance hierarchies in laboratory studies. Dominance behaviour usually correlated positively with metabolic rate, a phenomenon widely documented for salmonids (Burton et al., 2011). Our study was the first to investigate the relationship between metabolic parameters and rank position in the field. Contrary to the majority of laboratory-based studies, we did not find a relationship between metabolic rate and aggressiveness or rank position in the two

studied sites. As stated by Sloman et al. (2008), there is an enormous difference in complexity in social relations among animals confined in a tank compared to dominance hierarchies in the natural environment. Some studies showed that high metabolic rates are a key characteristic to achieve higher growth rates (Hoogenboom et al., 2013) and so, higher metabolic rate would be necessary for aggressive/dominant fishes to reach benefits in dominance. Although a relationship between metabolic rate and aggressiveness was not observed, aggressiveness was related with activity of electron transport system, a measurement of potential metabolic activity, i.e., level of oxygen consumption occurring at maximum enzymatic function (Muskó et al., 1995). However, this relationship occurred only in fishes from Dimona site. The residual analysis of ETS/RMR showed differences between animals from the two collecting sites. At Dimona site, dominant animals tend to have higher ETS/RMR rates than subordinates. A similar situation was found for animals from Amanã site. However, surprisingly, the most subordinate fish have also higher ETS/RMR rates at Amanã site. ETS/RMR ratio close to 1 means that respiratory activity is running at 100% capacity of ETS, and this suggests that the animal is close to its physiological limits (Bamstedt, 1980). Higher ETS/RMR is desirable as this indicates that the animal have larger energetic budget to be spent in all activities. So, the fact that dominant fish have higher ETS/RMR rates was expected, but the most subordinate fish also having higher ETS/RMR rates at Amanã site was not expected. Höjesjö, Johnsson, & Bohlin (2002) observed that the non-aggressive fish in the field have growth rate as higher as dominants and conclude that laboratory conditions can overestimate the fitness advantage of aggressive behaviour. These differences between study sites remain unclear. Differences in water flow,

379 dissolved oxygen or food availability between sites can affect physiological parameters
380 and consequently social behaviour and should be analysed in another opportunity.

381 Altogether, our results support the hypothesis that the dominant fish have a
382 higher metabolic scope when compared to subordinate fish. However, differences in
383 habitat characteristics may interfere with metabolic differences related to social position.
384 Further studies are needed to understand how environmental or population differences
385 promote physiological benefits to support a dominant position in dominance hierarchies.

386

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396

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507 **Table 1.** Aggression rate *versus* behavioral and physiological parameters in

508 *Apistogramma hippolytae* from Dimona and Amanã site. Rs= Spearman correlation

509 coefficient, P = significance probability

	Dimona		**Amanã**	
	Rs	P	*Rs*	p
Rank Position	**-0.951**	**<0.001**	**-0.963**	**<0.001**
Feeding rate	**0.393**	**0.03**	**0.658**	**<0.001**
Routine metabolic rate	-0.238	0.22	-0.217	0.32
ETS activity	**0.78**	**<0.001**	0.334	0.12
Rate ETS/R	**0.74**	**<0.001**	0.398	0.06

510

511

512 **Table 2**. Activity of electron transport system (ETS), routine metabolic rate (RMR) and

513 ETS/RMR *versus* wet body mass in *Apistogramma hippolytae* from Dimona and Amanã

514 sites. Rs= Spearman correlation coefficient, P = significance probability

515

	Dimona		**Amanã**	
	Rs	p	*Rs*	p
ETS activity	0.240	0.20	0.140	0.518
Routine metabolic rate	**-0.763**	**<0.001**	**-0.551**	**0.006**
Rate ETS/RMR	**0.633**	**<0.001**	0.372	0.08

516

517

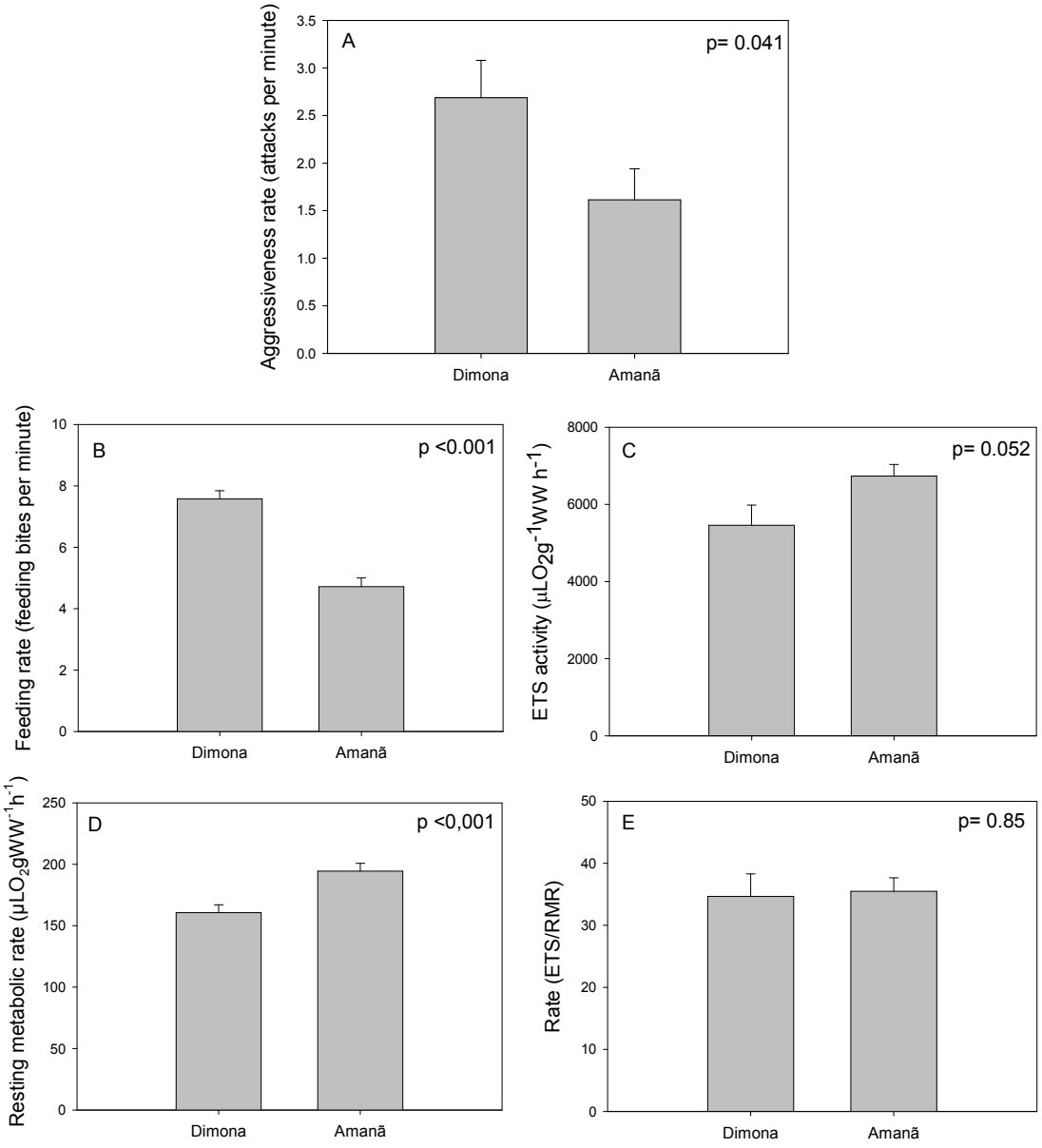
Figure captions

Figure 1. Aggressiveness rate (A), feeding rate (B), electron transport system (ETS) activity (C), routine metabolic rate (RMR) (D) and rate ETS/RMR (E) in *Apistogramma hippolytae* in Dimona and Amanã sites. (p) is the probability of averages being different after Student's t-test.

Figure 2. Observed *versus* predicted values for resting metabolic rate (RMR) and wet body mass in *Apistogramma hippolytae* from Dimona (A) and Amanã (B). No relationship with rank position was observed.

Figure 3. Observed *versus* predicted values for rate ETS/RMR and wet body mass in *Apistogramma hippolytae* from Dimona (A) and Amanã. In both sites, a tendency of higher rate ETS/RMR in dominant fish was observed. Note that fish from the lower social rank in Amanã site also have a high ETS/RMR rate.

531 Fig. 1



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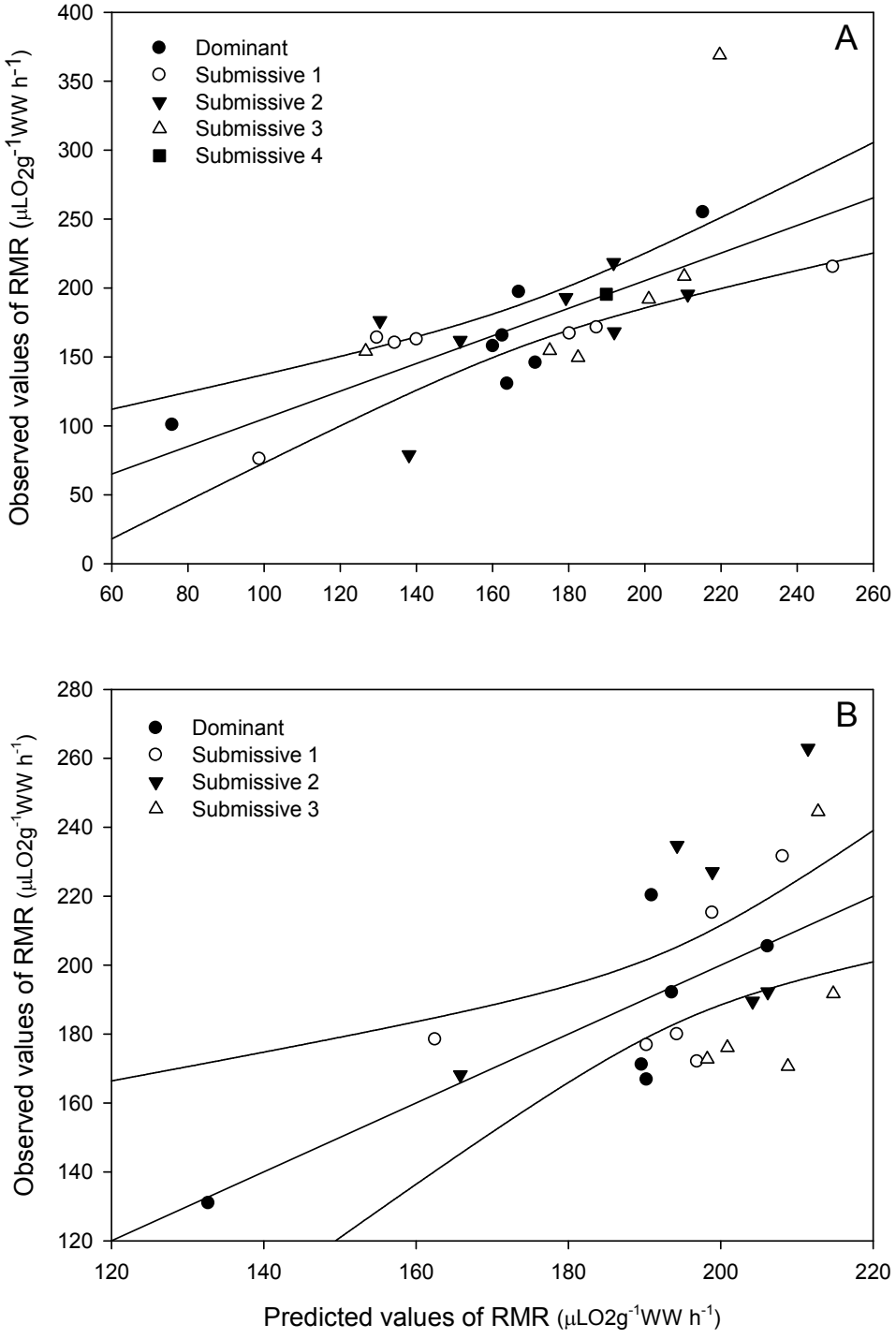
534 Fig. 2



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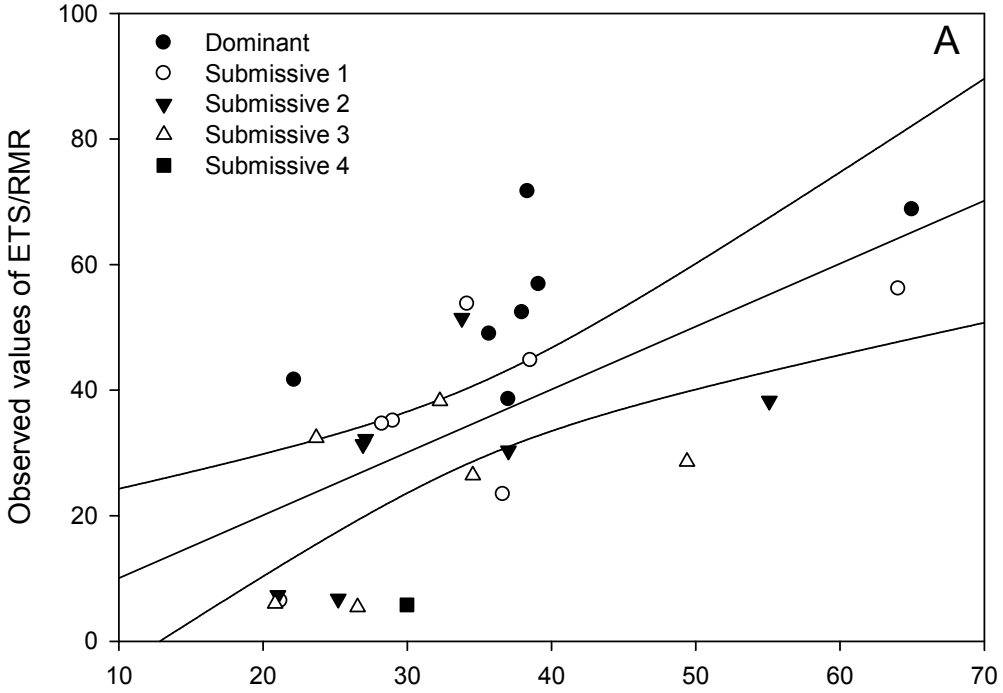
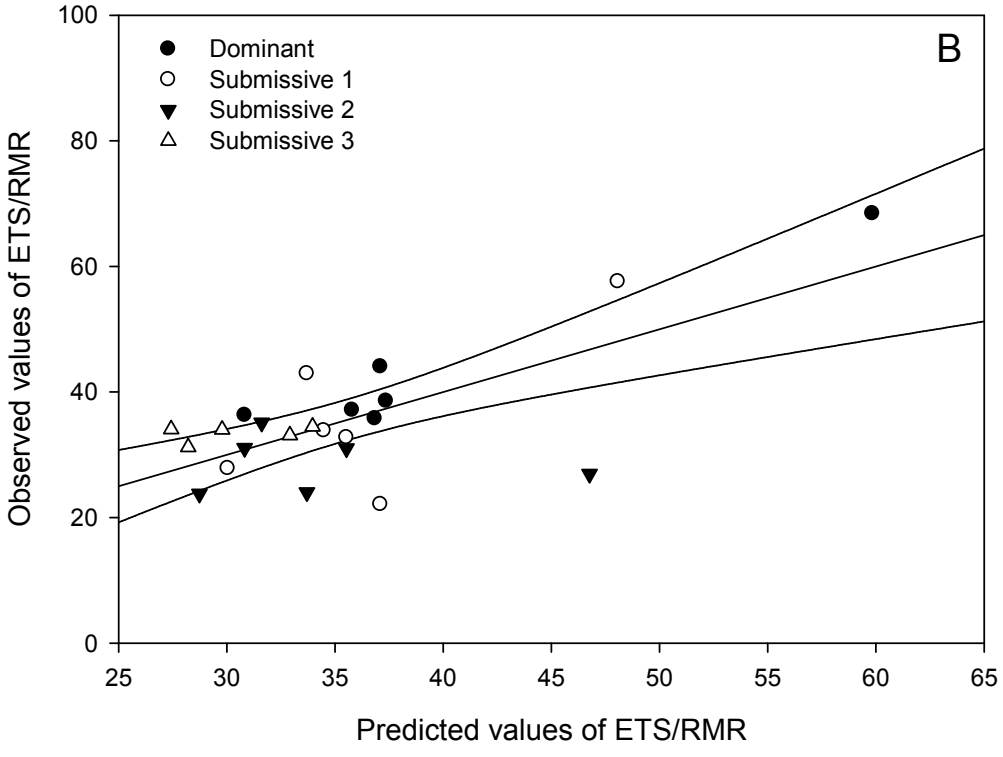
537 Fig. 3


A
Dominant
Submissive 1
Submissive 2
Submissive 3
Submissive 4
Observed values of ETS/RMR
0
20
40
60
80
100
10
20
30
40
50
60
70


B
Dominant
Submissive 1
Submissive 2
Submissive 3
Observed values of ETS/RMR
0
20
40
60
80
100
25
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35
40
45
50
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60
65
Predicted values of ETS/RMR

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SÍNTESE

Em conjunto, os resultados apresentados nos três capítulos mostraram a influência do status social no metabolismo aeróbico de duas espécies de ciclídeos do gênero *Apistogramma*, *A. agassizii* e *A. hippolytae*, especialmente na taxa metabólica de rotina. Também ressaltou-se que o ambiente possui importante papel na determinação do grau de influência que o status social possui sobre o metabolismo aeróbico das duas espécies. Nos dois primeiros capítulos, realizamos uma abordagem experimental em laboratório, isolando fatores ambientais, manipulando-os e observando as respostas em *A. agassizii*. O terceiro capítulo nos permitiu observar diferenças comportamentais e metabólicas entre duas populações naturais de *A. hippolytae*.

Por meio do estudo realizado em laboratório, enfatizamos a importância da estabilidade ambiental na manutenção das características das hierarquias de dominância. Mudanças ambientais como o aumento da temperatura e a diminuição do oxigênio afetaram a estabilidade e as características das hierarquias de dominância em *A. agassizii*. Todas as características avaliadas (agressividade, uso de abrigo e taxa de alimentação) sofreram a influência das variações nos parâmetros ambientais. Nosso estudo foi o primeiro a mostrar que mudanças nas hierarquias de dominância em ambientais instáveis envolvem principalmente na posição de dominância. Também observamos que apenas peixes dominantes de ambientes estáveis possuem uma taxa metabólica de rotina maior que peixes subordinados. Como o ambiente natural é normalmente variável, experimentando mudanças constantes na temperatura e nos níveis de oxigênio dissolvido, a estabilidade e características das hieraquias de dominância em ambientes naturais podem ser afetadas.

Também observamos que a complexidade estrutural do habitat interfere nas características das hierarquias de dominância. Embora não tenhamos observado um aumento geral na agressividade, observamos um aumento em um dos comportamentos agressivos, a taxa de mordidas dos dominantes nos subordinados foi maior no ambiente com maior complexidade estrutural quando comparado ao ambiente com reduzida ou sem complexidade estrutural. Esse aumento na taxa de mordidas

pelos dominantes observado nos ambientes mais complexos resultou em um aumento na taxa metabólica dos peixes dominantes, que também só foi observada nos ambientes mais complexos. Esse resultado corrobora estudos prévios que mostraram um aumento na taxa metabólica em peixes dominantes (Grantner and Taborsky, 1998; Metcalfe et al., 1995; Vollestad and Quinn, 2003; Yamamoto et al., 1998), mas mostra que essa diferença depende do habitat.

Quando estudamos a organização social de *Apistogramma hippolytae* em ambiente natural, em duas localidades, nas Reservas Dimona e Amanã, observamos que a espécie forma hierarquias de dominância. Porém, as características dessas hierarquias também foram localidade-específicas. A taxa de agressividade e de alimentação foi maior na Reserva Dimona. Como os fatores genéticos não foram isolados, não podemos afirmar se essas diferenças foram devido aos ambientes (já que esses possuiam diferenças na temperatura, na concentração de oxigênio e na velocidade de corrente da água) ou relativas a diferenças populacionais. Porém, os estudos realizados em laboratório nos dão um forte indicativo de que as variações ambientais devem ter contribuído para as diferenças observadas. O metabolismo aeróbico sofreu a influência do status social dos indivíduos, sendo que nas duas localidades os peixes dominantes possuiam um maior escopo metabólico, indicando vantagens metabólicas em peixes dominantes. Porém, na Reserva Amanã o peixe mais subordinado do grupo apresentou o mesmo perfil metabólico que o peixe dominante.

De modo geral, nossos resultados suportam a hipótese de que peixes dominantes possuem uma maior taxa metabólica que peixes subordinados. Porém, enfatizamos que essas diferenças são altamente dependentes do ambiente, tanto dos parâmetros físicos-químicos, quanto das características estruturais. Isso reforça a necessidade de um bom controle dos parâmetros físico-químicos e ambientais ao realizarmos estudos comportamentais em laboratório. Além disso, como o ambiente natural é normalmente instável e variável, tanto nos parâmetros físico-químicos quanto estruturais, as vantagens da posição de dominância em uma hierarquia podem estar sendo superestimadas. Estudos de longo prazo, que analisem o quanto essas

mudanças nas hierarquias de dominância são transitórias, são importantes para avaliarmos a real vantagem da posição de dominância em uma hierarquia.

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